UNIVERSITY OF CAMBRIDGE

Physiological and pharmacological factors affecting sickle cell function

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under the supervision of

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This dissertation is submitted for the degree of Doctor of Philosophy

Downing College

University of Cambridge

August 2020

Declaration

I, hereby declare that my dissertation entitled *Physiological and pharmacological factors affecting sickle cell function* is the result of my own original research. The outcome of work done in collaboration except where specially indicated in the text. Any parts of the dissertation have not been submitted for any other qualification. This dissertation does not exceed the word limit of 60,000 in agreement with the Degree Committee for the Faculties of Clinical Medicine and Veterinary Medicine.

Chun-Yen Lu
Cambridge, August 2020

Abstract

Thesis title: Physiological and pharmacological factors affecting sickle cell function

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Sickle cell disease (SCD) is one of the most common autosomal-recessive genetic disorders affecting millions worldwide. The three most significant abnormal features of RBCs from SCA patients are haemoglobin (HbS) polymerisation, phosphatidylserine (PS) exposure and significantly increased cation permeability via the KCl cotransporter (KCC), and are critical to the pathogenesis of SCD. The outcome of these abnormal features usually results in altered red blood cell (RBC) fragility and rheology, increased viscosity with blockage of small capillary vessels, and changed RBC membrane permeability. This study aimed to explore novel aspects of these three features with respect to their modulation by physiological stimuli experienced *in vivo* and a major pharmacological modulator, protein phosphorylation. Various physiological stimuli such as oxygen tension (PO_2), rate of deoxygenation, pH, urea, anisotonicity and medium composition, play an important role in regulation of HbS polymerisation, PS exposure and KCC activity in HbSS RBCs. In addition, low potassium (LK) sheep RBCs were utilised as an animal model to examine these physiological stimuli on KCC activity in comparison with their effects on human HbSS RBCs'. Various pharmacological manoeuvres were also tested for their effects on HbS polymerisation, PS exposure and KCC activity. A key finding was the marked stimulatory effect of the pan "with no lysine" kinase inhibitor WNK463 on KCC activity in HbSS RBCs, indicative of a critical role for WNK kinases in control of KCC in RBCs. However, there was no pharmacological evidence for the downstream involvement of SPAK/OSR1, although definitive evidence awaits phosphoproteomic studies. Overall, results provide a better and more comprehensive understanding of the pathogenesis of SCD with the potential to inform novel therapeutic approaches.

Dedication

I dedicate this thesis to my family and friends for their continuous support and encouragement made this work possible.

"It is the working man who is the happy man.
It is the idle man who is the miserable man."

- Benjamin Franklin

Acknowledgments

I would like to thank my supervisor Prof John Gibson for his kindness, patience and helpful suggestions throughout the years of my PhD program. I am also grateful to Dr Anke Hannemann, Dr Halima Al Balushi and my fellow doctoral student Mr Rasiqh Wadud for their help and advice.

My sincere thanks go to the medical team at the outpatient sickle cell clinic at King's College Hospital, London: Dr John Brewin and Prof David Rees, who kindly provide blood and clinical data weekly. I would also like to thank all the staff at the Department of Veterinary Medicine for providing a brilliant environment throughout my PhD.

I would like to thank Dr Joy Archer, Dr Lucia Sanchini, Dr Cassia Hare and Dr Maciej Guzera from The Queen's Veterinary Hospital for technical support.

Finally, I would like to acknowledge the support of my family and their patience throughout my PhD. My sincere thanks also go to my friends: Dr Wen-Yi Tseng; Mr Yung-Ching Wang's (founder of Formosa Plastics Group) and Mr Yung-Tsai Wang's family and their relatives; founder of Inventec Co Mr Shih-Chi Wen's family; Mr Nelson Chang (CEO of Techman Robot and executive manager of Quanta Computer) and all of my friends from Cambridge Taiwanese Society.

Abbreviation list

$[Ca^{2+}]_i$	Intracellular calcium concentration
[HbS]	Haemoglobin S concentration
A23187	Divalent cation ionophore
ATP	Adenosine tri-phosphate
BBS	Bicarbonate buffered saline
BEL-A cell lines	Bristol erythroid line adult
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
CCC	Cation-chloride cotransporters
cf.	Confer
Cl-MBS	Chloride MOPS buffered saline
CO_2	Carbon dioxide
DBP oil	Dibutyl phthalate oil
DeoxyHb	Deoxygenated Hb
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetra acetic acid
Fe^{2+}	Ferrous cation
Fe^{3+}	Ferric cation
Glu	Glutamic acid
Hb	Haemoglobin
HbA	Normal adult haemoglobin
HbAS	Haemoglobin S trait
HbF	Fetal haemoglobin
HbS	Sickle haemoglobin
HCO_3^-	Bicarbonate
Hct	Haematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	High potassium HEPES-buffered saline
HNO_3	Nitric acid

HU	Hydroxyurea (Hydroxycarbamide)
ISCs	Irreversibly sickled cells
K^+	Potassium ion
K_3FeCN_6	Potassium ferricyanide
KCC	Potassium chloride cotransporter
KCH	King's College Hospital
KCl	Potassium chloride
KNO_3	Potassium nitrate
Lys	Lysine
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
MOPS	3-(N-Morpholino) propane sulfonic acid
N_2	Nitrogen
Na^+	Sodium ion
NaCl	Sodium chloride
N-MBS	MOPS buffered nitrate solution
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
NEM	*N*-ethylmaleimide
NKCC	Na^+-K^+-$2Cl^-$ cotransporter
O_2	Oxygen
OSC	Oxygen saturation curves
OSR1	Oxidative stress response kinase 1
OxyHb	Oxygenated Hb
P_{50}	Oxygen tension required for 50% O_2 saturation of Hb
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
pH	Negative logarithm of the hydrogen ion concentration
PI	Phosphatidylinositol
PMCA	Plasma membrane calcium pump
PS	Phosphatidylserine
P_{sickle}	Deoxygenation-induced cation conductance

RBCs	Red blood cells
RuR	Ruthenium red
SD	Standard deviation
S.E.M.	Standard error of the mean
SCA	Sickle cell anaemia
SCD	Sickle cell disease
SM	Sphingomyelin
SPAK	SPS1-related proline/alanine-rich kinase (SPAK or STK39)
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
Triton™ X-100	Polyethylene glycol tert-octylphenyl ether
Val	Valine
WHO	World Health Organisation
WNK kinases	"With no lysine (K)" kinases

List of publications resulting from this work

i Articles in journals

1. Lu, D., Hannemann, A., Wadud, R., Rees, D., Brewin, J., Low, P. and Gibson, J., 2019. The role of WNK in modulation of KCl cotransport activity in red cells from normal individuals and patients with sickle cell anaemia. *Pflügers Archiv - European Journal of Physiology*, 471(11-12), pp.1539-1549.
2. Al Balushi, H., Dufu, K., Rees, D., Brewin, J., Hannemann, A., Oksenberg, D., Lu, D. and Gibson, J., 2019. The effect of the antisickling compound GBT1118 on the permeability of red blood cells from patients with sickle cell anemia. *Physiological Reports*, 7(6), p.e14027.
3. Gibson, J., Wadud, R., Lu, D., Brewin, J. and Rees, D., 2019. Oxidative stress and haemolytic anaemia in dogs and cats: a comparative approach. *Integrative Journal of Veterinary Biosciences*, 3(3).
4. Lu, D., Wadud, R., Hannemann, A., Rees, D. and Gibson, J., 2020. Effect of media composition on KCl cotranport activity in low potassium-containing sheep red cells. (in progress).
5. Wadud, R., Lu, D., Brewin, J. and Gibson, J., 2020. Pathophysiological relevance of renal medullary conditions on the behaviour of sickle cells. (in progress).

ii Posters

1. Lu, D., Hannemann, A., Rees, D., Brewin, J., Low P. and Gbison, J. (2018). WNK kinases and the activity of red cell KCl cotransport. Cambridge Science Festival, March 12 – 25, 2018, Cambridge, U.K.
2. Lu, D., Wadud, R., Hannemann, A., Rees, D. and Gibson, J. (2020). Effect of media composition on KCl cotranport activity in low potassium-containing sheep red cells, European Red Cell Society, September 11, 2020, Pavia, Italy.
3. Wadud, R., Hannemann, A., Lu, D., Rees, D., Brewin, J. and Gibson, J. (2020). PIEZO1-mediated Ca^{2+}-induced phosphatidylserine exposure in red cells from patients with sickle cell anaemia, European Red Cell Society, September 11, 2020, Pavia, Italy.

4. Lu, D., Wadud R., Hannemann, A., Rees, D., Low, P., Gibson, J., (2020). Regulation of red cell $Na^+K^+2Cl^-$ cotransporter and K^+Cl^- cotransporter and its relevance, American Red Cell Club, October 2020, Long Island, USA.

Table of Contents

1 Introduction

1.1 Sickle cell disease

The discovery of sickle cell disease (SCD) in Western medicine can be traced back to 1910. A dental student Walter Clement Noel, from the Caribbean island of Grenada, was diagnosed with pulmonary symptoms by two Chicago physicians, Dr James Herrick and Dr Ernest Irons (Herrick, 2014). Irons firstly described Noel's red blood cells (RBCs) as "having the shape of a sickle", which then inspired the cardiologist Herrick to consider that it might be a disease sui generis. Herrick then published a paper in the Yale Journal of Biology and Medicine, coining the term "sickle-shaped cells". In Herrick's clinical report, he offered only sketchy details of a characteristic feature of SCD: "elongated, sickled cells" (Herrick, 2014). However, this clinical report still quickly drew Western clinicians' attention. Later, it was defined as a genetic disease in which most patients had the homozygous genotype HbSS rather than HbAA of normal people. Moreover, heterozygotes AS (HbAS) patients (also called sickle trait individuals) appeared to be relatively asymptomatic although their RBCs could still be induced to become sickled *ex vivo*.

In 1949, the Nobel prize laureate Dr Linus Pauling and his colleague Dr Harvey Itano discovered that haemoglobin had different biological properties in SCD patients. They utilised the cutting-edge biotechnology of that time, gel electrophoresis, to examine the difference of haemoglobin mobility among SCD individuals, sickle trait individuals and normal people. The differences in mobility which were obtained, indicated charge differences and therefore an altered haemoglobin structure of HbS compared to that of HbA. The disease was then defined by Linus Pauling in his paper, entitled "Sickle cell anemia, a molecular disease" (Pauling *et al.*, 1949).

Since then, the pathophysiological mechanism of SCD has been gradually unmasked and various novel therapeutic approaches have been devised to treat SCD.

1.2 Epidemiology

SCD was originally confined to the tropics and subtropics, where it was endemic in many countries, but is now common worldwide due to globalisation and migrations. Nowadays, approximately 5% of the world's population carries genes for haemoglobin disorders, the most common of which are SCD and thalassemia. The global distribution of haemoglobin disorders is shown in Figure 1.1. The most common areas for SCD patients are Africa, the Middle East, South America, Central America, India and some Mediterranean countries (Greece, Turkey and Italy). According to a World Health Organisation (WHO) report in 2008 and Piel *et al.*'s report in 2010, more than 350,000 new-born babies suffer from a severe haemoglobin disorder (including SCD and thalassemia) each year. Approximately 80% of affected children are born in poor or developing countries, with a lethality of 3.4% in children aged under five years old.

Geographically, due to globalisation, approximately 50 million heterozygous HbS individuals have spread throughout the world nowadays, being found in 290 countries. Evidence also indicates that more than 9 million carriers become pregnant annually, thus increasing the number of people carrying the sickle gene (Modell and Darlison, 2008; Piel *et al.*, 2013). In Africa, approximately 10% – 40% of the population carries the HbS mutation. In the U.S., the incidence of SCD patients is 1 SCD new-born baby in 600 births in the African-American population (African heritage). SCD affects approximately 60,000 – 70,000 people living in the U.S. In France, there were 405 new-born SCD babies in 2007 and a total of approximately 10,000 people were reported to be SCD patients (De Montalembert & Davies, 2001).

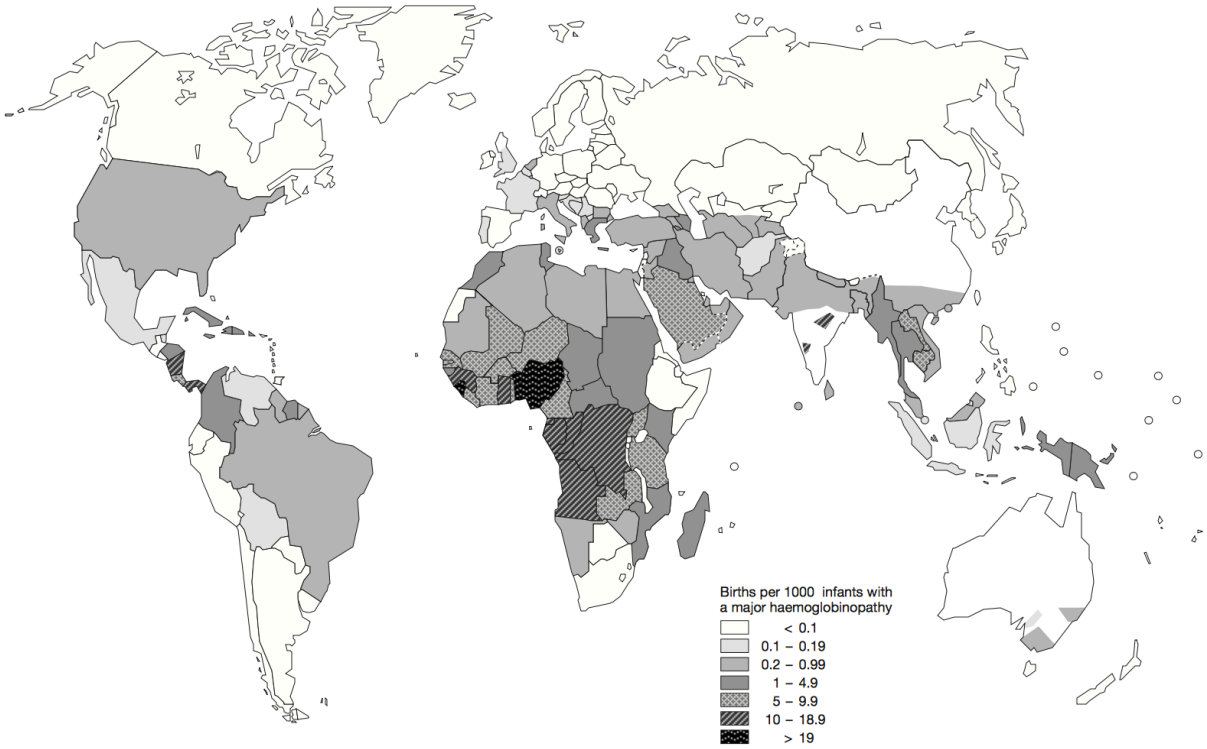

Figure 1.1 Global distribution of pathological haemoglobin disorders. The people living in the darkest area are suffering from more than 19 affected new-born babies per 1,000. The rest of the world presents a reduced prevalence of SCD. Haemoglobin disorders including sickle cell disease are usually found in poor and developing countries partly due to the absence of prenatal diagnosis. SCD patients are mainly found in Africa, Middle East, South America, Central America, India and some Mediterranean countries, where it represents a severe inherited public disease (Weatherall, 2010).

1.3 Pathophysiology

1.3.1 Aetiology

In normal adult human RBCs, the molecular weight of one HbA is 64 kDa, existing as a tetrameric 2α2β polypeptide globin chain. In the centre of each polypeptide chain, Fe^{2+} is embedded in a heme group within a porphyrin ring, making the haem molecule able to bind to an oxygen (O_2) molecule reversibly (Steinberg, 1998). HbA functions as an oxygen-binding metalloprotein, delivering O_2 from lung to other organs of the human body via the cardiovascular system (Sidell & O'Brien, 2006). The structure of Hb can be changed by binding of O_2 to each of the four Fe^{2+}. Under hypoxic environment, Hb becomes deoxygenated, which is in the "tense shape" (T), rather than the "relaxed shape" (R) in oxygenated environment. Some other gases such as carbon dioxide (CO_2) and nitric oxide (NO), which also have various physiological functions, are also carried by haemoglobin throughout the human body.

SCD arises from a gene defect on the short arm of chromosome 11 (11p15.5), which encodes β haemoglobin, and contains a point mutation (GAG to GTG) which changes the amino acid from glutamic acid (negative charge) to valine (uncharged) at the 6th amino acid of the β chain, enabling a hydrophobic interaction with another haemoglobin molecule. Under the deoxygenated environment, the presence of HbS causes a tendency of haemoglobin to polymerise to form an elongated rope-like fibre, which distorts the morphology of RBCs into sickle shape (C-shape), resulting in RBCs lesions. The accumulated rigid RBCs are responsible for the complications of SCD such as vaso-occlusion and acute chest syndrome which are hallmarks of the disease (Pauling *et al.*, 1949; Ingram, 1957; Marotta *et al.*, 1977).

1.3.2 HbS polymerisation, crystal-solution equilibrium and sickling

As referred to in section 1.3.1, HbS molecules in SCA patients aggregate and form 14-membered polymers or fibres under hypoxic environment, in which valine from one HbS molecule binds to leucine, phenylalanine and alanine from an adjacent HbS molecule by forming hydrophobic bonds (Ingram, 1957). There are physiological factors which may alter the formation of HbS: high intracellular [HbS], oxygen tensions, low pH, urea, low Mg^{2+} and high temperature (Eaton & Bunn, 2017).

The polymerisation of sickle haemoglobin is an equilibrium between insoluble HbS crystals and dissolved HbS in solution. The kinetic features of HbS polymers formation underlie the critical determinants of sickle RBCs morphology. There is a delay time (T_D) for the formation of haemoglobin polymers following deoxygenation. Polymerisation consists of a two-pathway mechanism including homogeneous nucleation and heterogeneous nucleation (Ferrone *et al.*, 1980). Homogenous nucleation occurs when haemoglobin polymers form through an unstable polymerisation, followed by the second pathway, the heterogeneous nucleation, which is located on the surface of these initial polymers (Ferrone *et al.*, 1980) (Figure 1.2). Robust evidence indicates that SCD would become a milder disorder in the presence of only homogeneous nucleation (Bunn, 1997). Moreover, if HbS in sickle RBCs does not undergo heterogeneous nucleation events, deoxygenated sickle cells will take a longer time to form fully sickled RBCs and the delay can be enough for cells to return to the lungs before forming insoluble polymers (Mirchev & Ferrone, 1997). If sickle RBCs are exposed to a profoundly hypoxic environment, homogeneous nucleation is favoured, and the haemoglobin polymerisation events will result in a granular or cobblestone texture, which does not distort the biconcave shape of HbS-containing RBCs as much and has a milder disorder. In contrast, if HbSS RBCs are slowly deoxygenated or exposed to less profound hypoxia, haemoglobin S fibres will form in RBCs through both nucleation events, thereby altering the shape of RBCs to a greater extent, producing more severe complications (Mozzarelli *et al.*, 1987).

Moreover, some sickle HbS RBCs do not depolymerise when re-oxygenated. These RBCs are believed to have membrane protein and cytoskeletal abnormalities and are referred to as irreversibly sickled cells (ISCs). ISCs are more prone to haemolysis than the reversible sickle RBCs (Serjeant, 1970).

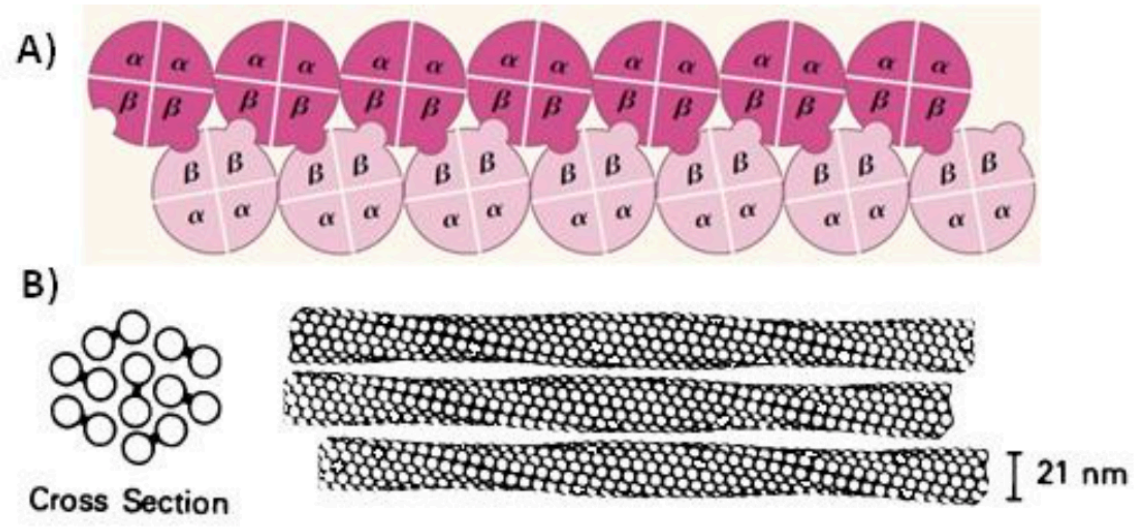

Figure 1.2 The formation of HbS polymer: (A) in the deoxygenated state, adjacent HbS molecules bind to each other via their β chains (Bunn, 1997); (B) cross section of HbS polymer presenting as 7 double-stranded helical structures which form a rope-like structure (Eaton & Hofrichter, 1990).

1.4 Phosphatidylserine (PS) exposure in SCD

Normally, the regular life span of human RBC is about 120 days, after which they become old and can be eliminated phagocytically by macrophages. In SCD patients, the life span of their RBC is much shorter at about 10-20 days, leading to severe complications such as stroke, acute chest syndrome and pulmonary hypertension, etc (McCurdy & Sherman, 1978; Steinberg, 2008; Rees *et al.*, 2010; Chakravorty & Williams, 2015). In the healthy RBC membrane, the aminophospholipids phosphatidylethanolamine (PE) and phosphatidylserine (PS) are in the inner side of RBC membrane, while the sphingomyelin (SM) and phosphatidylcholine (PC) are on the outer side of membrane bilayer (Kuypers, 2007). More types of phospholipids such as phosphatidylinositol (PI) and cholesterol play an important role in providing RBC plasma membrane functional fluidity and asymmetry, which in turn ensures stability and integrity of RBCs. The negatively charged PS, when exposed on the red cell surface, could allow adherence of other cell types such as endothelial cells and macrophages during microcirculation. A glycoprotein secreted by macrophages, lactadherin (LA), provides a PS-binding motif for the detection of PS-expressing RBCs (Verhoven *et al.*, 1995; Dasgupta *et al.*, 2008; Leventis & Grinstein, 2010) (Figure 1.3). Moreover, the externalised PS can also be recognised by an adhesion receptor expressed by activated endothelia cells (Yamaja Setty *et al.*, 2002; Yamaja Setty &

Betal, 2008). Overall, PS exposure can be seen in some haematopoietic cells such as RBCs, lymphocytes and platelets, which is an early signal of programmed cell death (Zwaal & Schroit, 1997; Zwaal *et al.*, 2005). As one of the hallmarks of SCD, PS exposure in HbSS RBCs is 2-11% higher than that in healthy human RBCs (Kuypers *et al.*, 1996; Wood *et al.*, 1996; De Jong *et al.*, 2001).

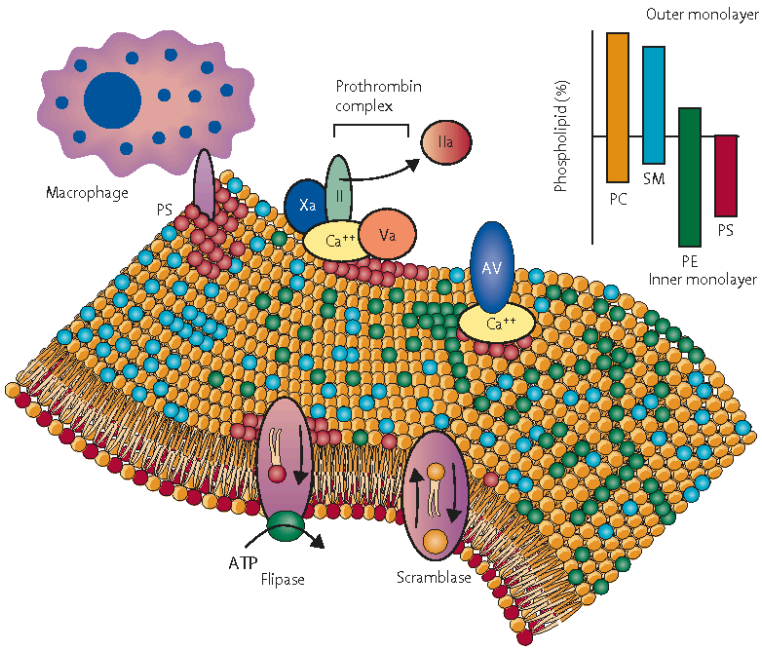

Figure 1.3 Schematic diagram of the phospholipid composition of RBC membrane containing with two important membrane transport systems: flippase and scramblase. The Ca^{2+}-activated coagulation cascade (factor Va, Xa and II) and phosphatidylserine (PS) is attractive to macrophages. SM: Sphingomyelin, PC: Phosphatidylcholine, PE: phosphatidylethanolamine, PS: Phosphatidylserine (Stuart & Nagel, 2004).

The mechanism of the externalisation of PS still remains unidentified. However, the deoxygenation-induced HbS polymerisation and sickling shape change has been suggested to play a role in inducing PS exposure of HbSS RBCs (Lubin *et al.*, 1981; Blumenfeld *et al.*, 1991; De Jong *et al.*, 2001). Two membrane transport systems, which are the aminophospholipid translocase (APLT or flippase) and the scramblase (phospholipid scramblase), are responsible for maintaining the normal phospholipid distribution (Figure 1.3). The flippase, the first transport system, is an ATP-dependent primary active transport system, which is homologous to the P-type ATPase and a subfamily of P_4-ATPase, functioning as transporting PE and PS from the outer membrane to the inner membrane (Seigneuret & Devaux, 1984; Daleke & Lyles, 2000;

Daleke, 2007). The second transport system, the scramblase (PLSCR, or phospholipid scramblase), is suggested as a phospholipid transporter, which can be activated by Ca^{2+} and moves lipids bidirectionally (Barber *et al.*, 2009). Normally, the scramblase remains inactivated in healthy RBCs. However, the scramblase becomes activated in the presence of Ca^{2+}, whose entry is mediated by P_{sickle} (see later), suggesting a role of Ca^{2+} in the mechanism of PS exposure. Pharmacologically, the ionophore Br-A23187 has been widely utilised in making RBC membrane highly permeable to Ca^{2+} (Bevers & Williamson, 2010). Owing to this method, the free $[Ca^{2+}]_i$ can be fixed and calculated by utilising the Donnan ratio (Flatman, 1980; Muzyamba *et al.*, 2006).

Since then, studies have proposed that Ca^{2+} may activate the scramblase and inhibit the flippase. The scramblase can activated when $[Ca^{2+}]$ is about 100 μM *in vitro* (Kamp *et al.*, 2001), although more recent estimates indicate a much higher Ca^{2+} affinity of a few micromolar (Weiss *et al.*, 2012; Cytlak *et al.*, 2013), while the flippase can also become inhibited when $[Ca^{2+}]$ is about 0.2-1 μM *in vitro* (Bitbol *et al.*, 1987). The localisation of PS is partially determined by the ratio of activities of flippase to scramblase (F/S ratio), with a high F/S ratio leading to the internalisation of PS and *vice versa* (Barber *et al.*, 2009). In HbSS RBCs, the Gardos channel also becomes activated with a Ca^{2+} threshold of about 100 nM (Lew & Bookchin, 2005; and see later), leading to cell dehydration and further PS exposure under hypoxic environment.

1.5 Pathways of abnormal transport in sickle RBCs

Isosmotic dehydration of sickled RBCs *in vivo* may be the consequence of the activation of one or various transporters located on RBCs plasma membranes. The predominant transport systems are represented by: (1) the deoxygenation-induced cation pathway (P_{sickle}); (2) the Ca^{2+} -activated K^+ channel of intermediate conductance (or Gardos channel), which is cloned and is represented by IK1 (hSK4) (Hoffman *et al.*, 2003) and (3) the K^+-Cl^- cotransporter (KCC) (Figure 1.4).

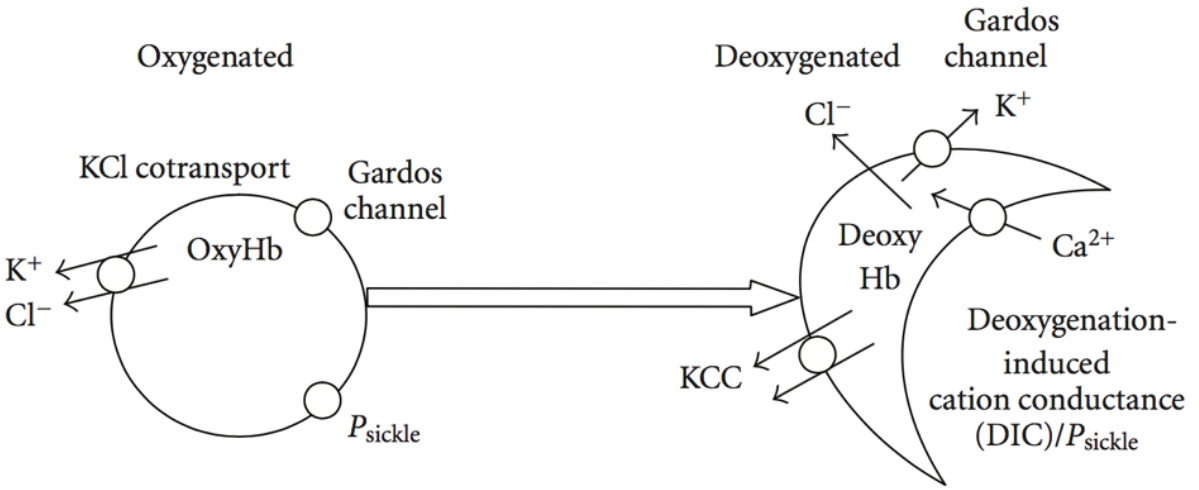

Figure 1.4 Schematic diagram of the three transport systems involved in dehydration of sickle RBCs. High cation permeability occurs in sickle RBCs caused by three highly active transport pathways. In the oxygenated sickle RBCs, KCC remains active and induces the loss of K^+ and Cl^-; P_{sickle} and Gardos channel remain relatively quiescent. In deoxygenated RBCs, KCC is still active; P_{sickle} and Gardos channel are also activated. P_{sickle} is deoxy-dependent and activated by the polymerisation of HbS and plays an important role in mediating the entry of Ca^{2+}. The Gardos channel is then activated through increased concentration of intracellular Ca^{2+}, which further increases the concentration of HbS and promotes HbS polymerisation (taken from Hannemann *et al.*, 2011).

1.5.1 The deoxygenation-induced cation pathway (P_{sickle})

P_{sickle} is triggered by deoxygenation of sickle RBCs. Nowadays, P_{sickle} is described as an unidentified non-specific cation channel(s) which is/are activated physiologically (Ranney, 1997). Several important works carried out by Joiner and colleagues tried to elucidate the role of P_{sickle} in mediating the entry of Na^+ and the loss of K^+ in sickle RBCs (Joiner *et al.*, 1986). They primarily found out that under the circumstances of PO_2 less than 40 mmHg, supplemented with extracellular divalent cations, the loss of K^+ is greater than the entry of Na^+, representing the major cause of RBC dehydration (Joiner *et al.*, 1995). Some early research was carried out by Lew and his colleagues, who endeavoured to elucidate the nature of P_{sickle} in 1990s. After carefully comparing HbSS RBCs with normal RBCs, they found out that the reason for dehydration of sickle cells is the enhanced permeability of Ca^{2+} in HbSS RBCs. They observed that the dehydration of sickle cells was deoxy-dependent with the presence of P_{sickle} in dense / dehydrated reticulocytes (Bookchin *et al.*, 1991). Moreover, to restore the balance of Na^+ and K^+, Na^+/K^+ pump (3:2 Na^+/K^+ flux ratio) consumes energy by hydrolysing ATP and then leads to further solute loss (Post *et al.*, 1967; Joiner *et al.*, 1986).

To summarise, the existence of P_{sickle} will enhance the entry of Ca^{2+}, and the loss of some other cations such as Mg^{2+} in sickle RBCs in the deoxygenated state. The entry of Ca^{2+} plays a particularly important role in activating the second transport pathway, the Gardos channel (Rhoda *et al.*, 1990). Loss of Mg^{2+} via P_{sickle} may also be important in activating KCC through alteration of the balance of kinase and phosphatase activities which are implicated in its regulation - see later.

1.5.2 The Gardos channel

The Gardos channel of human RBCs was first discovered by Györgi Gárdos in 1958 (Gárdos, 1958). The Gardos channel in RBCs plasma membrane, belongs to a family of Ca^{2+}-activated K^+ channels of intermediate conductance. It is encoded by *KCNN4* gene. The Gardos channel ubiquitously exists on the membrane of RBCs and approximately 150 channels exist per mature erythroid cell (Wolff *et al.*, 1988). Due to the high capacity of the Gardos channel, it functions as a gatekeeper of calcium-activated potassium efflux. In various cell lines and tissues, it can mediate normal and pathological roles such as immunologic function, cardiovascular system, asthma and neuroprotection (Christophersen & Wulff, 2015).

Owing to inherent difficulties, it is currently difficult to crystallise the Gardos channel, with implicit problems for analysis of its structure. Conventional methods to analyse Gardos channels, such as NMR spectroscopy and X-ray crystallography, only provide a potential structure. Hence, the structure of the Gardos channel is predominantly deduced from the known structures of other similar proteins (Vandorpe *et al.*, 1998). As shown in Figure 1.4, the basic structure of the Gardos channel is thought to comprise the six transmembrane domain (TMD) polypeptides (s1 – s6 domains), accompanied by one "pore" region. The channel in the RBCs plasma membrane exists as a homotetramer (Doyle *et al.*, 1998). The major functional motif of Gardos channel are the "p" regions, which function as the selectivity filter and pore. The movement of K^+ is mediated by the carbonyl oxygen atoms within the "p" region protruding from the backbone of the polypeptides from each of the four identical subunits.

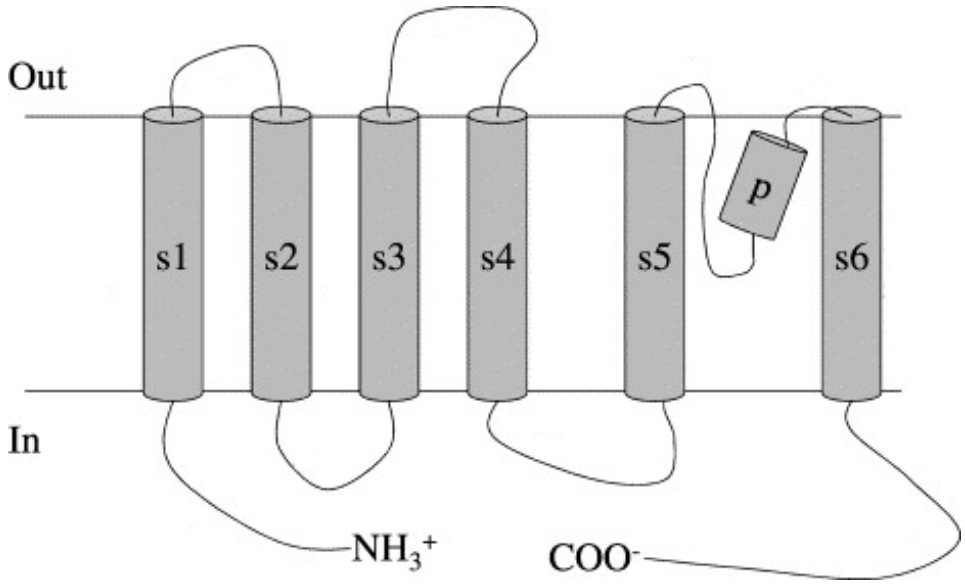

Figure 1.4 The structure of the Gardos channel. Six putative TMDs (s1-s6) and the "p" domain form the channel, which serves as selectivity filter and pore (Vandorpe *et al.*, 1998). The main functional entity of the channel is predicted as a tetramer (Vandorpe *et al.*, 1998).

In normal HbAA RBCs, the intracellular concentration of Ca^{2+} (~20-50 nM) is kept low by the plasma membrane calcium pump (PMCA, in human isoforms of PMCA1 and PMCA4), at which the Gardos channel is normally inactive (Lew *et al.*, 1982; Gibson & Ellory, 2002). However, in sickle cells, the Gardos channel is activated by elevated [Ca^{2+}] (~100-150 nM, the entry of Ca^{2+} is via P_{sickle} transport pathway) (Bennekou *et al.*, 2004; Lew & Bookchin, 2005). Robust evidence shows that highly activated Gardos channels in RBCs mediate a marked loss of K^+ with Cl^- and water, leading to RBC shrinkage, [HbS] elevation and acceleration of HbS polymerisation (Brugnara *et al.*, 1993; Etzion *et al.*, 1993).

1.5.3 The KCl Cotransporter (KCC)

KCCs are expressed in a large variety of cells such as RBCs, tumour cells and hepatocytes, which have the potential to mediate regulatory volume decrease (RVD) following cell swelling (Lauf *et al.*, 1992; Shen *et al.*, 2003). In 2005, Crable *et al.* indicated that there are three KCC isoforms in the RBC membrane, which are KCC1 (SLC12A4, on chromosome 16q22), KCC3 (SLC12A6, on chromosome 15q14) and KCC4 (SLC12A7, on chromosome 5p15) (Crable *et al.*, 2005). All three KCC isoforms have twelve TMDs, some N-glycosylation sites occur between TMD-5 and TMD-6, and cytoplasmic amino and carboxyl terminal domain (Figure 1.5). The TMD-2 plays a role

in mediating cation transfer (Isenring & Forbush, 2001; Bergeron *et al.*, 2006). In RBCs from normal individuals and SCD patients, KCC can mediate solute loss and alter cell volume (Quarmyne *et al.*, 2011). However, when normal individual RBCs become mature (aging), the activity of KCC progressively decreases but can still be partially activated by *in vitro* pharmacologically incubating with thiol-reactive agents such as *N*-ethylmalemide (NEM) (Lauf *et al.*, 1985). Its physiological role is probably to mediate the reduction in RBC volume as erythroblasts mature into erythrocytes.

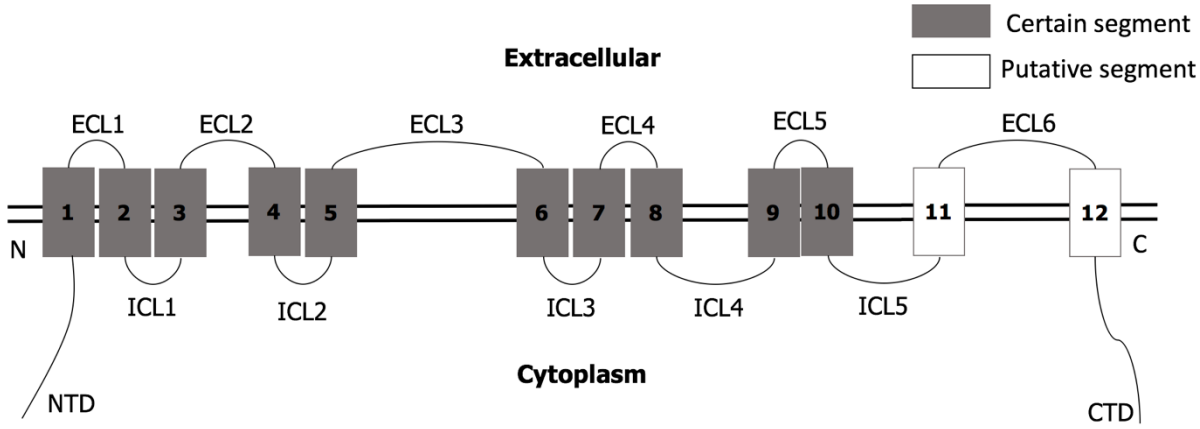

Figure 1.5 The structure of KCC. KCC has twelve TMDs. From domain 1 to domain 10 are definite segments (grey). Domain 11 and domain 12 are putative segments (white). NTD (N-terminal domain) and CTD (C-terminal domain) occur in the cytoplasm. ECL represents extracellular loop. ICL represents intracellular loop (Zhang *et al.*, 2005).

In sickle RBCs, abnormal KCC activation can induce the loss of intracellular potassium and chloride with water following osmotically via other channels (aquaporins), leading to RBCs shrinkage, [HbS] elevation and HbS polymerisation acceleration, which in turn induce the red cells to sickle (Quarmyne *et al.*, 2011). In SCD patients, the details of KCC activation are complex and remain unclear, with various possible physiological stimuli including low pH, urea, volume and Mg^{2+} depletion (Jennings & Al-Rohil, 1990). Moreover, it is also proposed that KCC is bound to the N-terminal of band 3 cytosolically, and that the activation of KCC might follow polymerisation of HbS and RBC shape distortion (Gibson & Ellory, 2002). Pharmacological evidence and molecular biology indicate that KCC is regulated by protein phosphorylation / dephosphorylation, via cascades of regulatory protein kinases and phosphatases such as protein kinases Syk and Src and phosphatases PP1 and PP2A (Bize *et al.*, 1999; Merciris *et al.*, 2003). Moreover, WNK/SPAK/OSR pathway has been suggested to inhibit KCC by

phosphorylation in HEK293 cell lines but also in red cells (Rinehart *et al.*, 2009). Also, some other serine-threonine protein phosphatase inhibitors such as NEM and okadaic acid have suggested to be potent physiological blockers of KCC (Jennings & Schulz, 1991). Overall, KCC activation has been concluded to act as a net dehydration event, which is associated with solute loss and water dehydration.

Another important feature of KCC in RBCs from SCD patients is its abnormal response to oxygen tension. In normal RBCs, KCC is inactivated at low oxygen levels, and the main stimuli associated with KCC activation – low pH and urea – are found is hypoxic regions. This means that KCC in normal RBCs cannot respond to these major physiological stimuli. In sickle cells, however, KCC activity at first reduces as oxygen tension is lowered, but at about the P_{50} for Hb saturation, KCC activity increases again, such that in fully deoxygenated RBCs, KCC activity is actually higher than in fully oxygenated ones (Gibson *et al.*, 1998, 2001). This is significant because it means that in sickle cells, KCC activity can be stimulated by low pH and urea even in hypoxic regions, and thereby mediate solute loss and RBC dehydration.

1.6 KCC in LK (low potassium) sheep RBCs

In 1954, Evans and colleagues found that the $[K^+]$ in sheep RBCs is genetically controlled being either high K^+-containing (HK) or low K^+-containing (LK) (Evans, 1954). In HK sheep RBCs, high activity of the Na^+/K^+ pump occurs on the RBC membrane together with a low passive K^+ leak; while conversely in LK sheep RBCs, there is no Na^+/K^+ pump activity, and a high passive K^+ leak (Tosten & Hoffman, 1960; Dunham, 1976). This phenomenon is the consequence of Mendelian inheritances, controlled by a single gene with two alleles, *l* and *m*, determining whether sheep RBCs become HK or LK (Evans and King, 1955; Rasmusen and Hall, 1966). Homozygous *mm* sheep have HK RBCs. In young sheep's RBCs or precursor RBCs, all RBCs also present as HK. Sheep of the *ll* genotype or heterozygous *lm* have LK RBCs when they are mature. RBCs from these animals have a particularly high activity for KCl cotransporter (Brown *et al.*, 1978; Dunham & Ellory, 1981). Hence, mature LK sheep is a good animal model for studying KCC, to characterise this Cl-dependent, volume-sensitive transport pathway and it has been much studied, making it a particularly useful model for understanding the behaviour of KCC in SCD (Bergh *et al.*, 1990).

1.7 Treatment of SCD

Since the discovery of SCD by James Herrick in 1910, the treatment of SCD remains largely supportive although there has been considerable effort to design more specific therapies. Hydroxyurea (hydroxycarbamide or HU) therapy has been invented and utilised to treat SCD from the 1995 onwards (Charache *et al.*, 1995). The mechanism of HU therapy is to enhance the transcription of the gamma (γ) chain of haemoglobin, which in turn increasing the level of fetal haemoglobin G (HbF) (its biological structure: $\alpha_2\gamma_2$ instead of $\alpha_2\beta_2$), attenuating the severity of SCD by preventing HbS molecules polymerisation (Watson, 1948; Covas *et al.*, 2004). Even so, the exact mechanism of HU therapy remains unclear. However, the HU therapy has been suggested to have some toxic side effects to human body and furthermore is teratogenic so that its use is largely confined to more severely affected SCD patients (Vasavda *et al.*, 2008; Rees, 2011; Strouse & Heeney, 2012). Traditionally, exchange transfusion and regular blood transfusion have been utilised for the SCD management. Though blood transfusion can attenuate the severity of SCD, tissue damage and some other complications in SCD patients may be caused by the resulting iron overload, haemorrhagic complications and blood transmitting infections (Embury, 1996; Agble *et al.*, 1998; Swerdlow, 2006). In addition, penicillin, an antibiotic drug, has been utilised as a complimentary disease management in SCD patients, especially to increase the survival rate of SCD children by preventing pneumococcal sepsis infection (Rees, 2011). However, there is an urgent need for developing new drugs or a combination of several drugs to increase the level of HbF but with milder side-effects than HU therapy or to attenuate the condition by some other mechanisms (Ataga, 2009).

More recently, some other treatment strategies have been investigated to control HbS polymerisation, prevent sickling, increase HbS oxygen affinity, increase the dehydration of RBCs, inhibit platelet activation, increase the level of HbF, inhibit Ca^{2+} channels, develop inhibitors for P_{sickle}, Gardos channel and KCC, reduce the adhesion of RBCs and provide antioxidants. A new drugs called voxelotor, an aromatic aldehyde derivative, functions as an inhibitor of HbS polymerisation through increasing the oxygen affinity of HbS, and is currently in phase III trial (Vichinsky *et al.*, 2019). Also, in 2017, the emergence of a new strategy of gene therapy has been utilised as well.

A 13 years old male patient showed positive results after transplanting him with autologous haematopoietic cells in which the cells were inserted with a lentiviral vector-mediated gene to replace HbS with normal Hb (an antisickling β-globin gene, $\beta^{A(T87Q)}$) (Ribeil *et al.*, 2017).

However, gene therapy is not affordable due to the high medical cost of transfecting haematopoietic cells whilst SCD is most prevalent in some developing countries with relatively low economic resources. Hence, there is an urgent need to find cheaper treatment strategies for some SCA patients. Some physiological stimuli treatment and pharmacological treatments have been the focus of recent studies and have shown some promising results.

1.8 Hypothesis and aims

The three most significant abnormal features of red cells from SCA patients, which contribute in a major way to pathogenesis are: PS exposure increased by deoxygenation and sickling; HbS polymerisation and sickling; and increased cation permeability notably via the KCl cotransporter (KCC) with its aberrant response to oxygen level. A common link between all three are their reactions to oxygen tension and the deleterious effects of deoxygenation.

This thesis will explore novel aspects of these three features with respect to their modulation by physiological stimuli experienced *in vivo* and a major pharmacological modulator, protein phosphorylation.

1.8.1 Specific hypotheses

1. Activation of HbS polymerisation and sickling; PS exposure; and KCC by key physiological stimuli and pharmacological modulators contributes significantly to the pathogenesis of SCD.
2. A better understanding of the mechanisms involved will inform the development of novel therapeutic drugs.

1.8.2 Specific aims

Chapter Four: Investigation of key physiological and pharmacological stimuli / modulators which may alter HbS polymerisation and sickling.

Chapter Five: Investigation of key physiological and pharmacological stimuli / modulators which may alter PS exposure.

Chapter Six: Investigation of key physiological stimuli / modulators which may alter KCl cotransport.

Chapter Seven: Investigation of key pharmacological stimuli / modulators which may alter KCl cotransport.

2 Materials

2.1 Sheep red blood cells (RBCs) samples

Adult sheep samples were purchased from TCS Biosciences Ltd. (Botolph Claydon, U.K.) and were stored at 4 °C in the refrigerator until needed. All experiments were carried out within 48 hours after blood collection.

2.2 Human blood samples

Human HbAA and HbSS blood sample were routinely collected by Professor David C. Rees from King's College Hospital, which is with ethical permission (Reference Numbers: 11/LO/0065 and UI/EC/13/0077). Blood was stored in EDTA-containing vacutainers, stored at 4 °C in the refrigerator until needed. All experiments using blood were carried out within 72 hours after blood collection.

2.3 Chemicals and solutions:

Table 2.1 List of chemicals and suppliers where they were purchased from. All chemicals were supplied by Sigma-Aldrich Chemical Co. (Poole, UK), unless otherwise stated.

Name	**Supplier**
Radioisotope	
$^{86}Rb^{+}$	Perkin Elmer (Beaconsfield, UK)
Inhibitors	
Bumetanide	Calbiochem (Nottingham, UK)
Ouabain	
Gas	
CO_2 gas cylinder	BOC Gases (Guildford, UK)
N_2 gas cylinder	BOC Gases (Guildford, UK)
O_2 gas cylinder	BOC Gases (Guildford, UK)
Pharmacological reagents	
Calyculin A	
Cytochalasin B	
Ethylenediaminetetraacetic acid (EDTA)	
GsMTx-4	Abcam (Cambridge, UK)
N-ethylmaleimide (NEM)	
Ruthenium Red (RuR)	
Staurosporine	
WNK463	
Other reagents	
3-(N-morpholino) propanesulfonic acid (MOPS)	
Br-A23187 ionophore	
Calcium chloride ($CaCl_2$)	
Dibutyl phthalate (DBP) oil	
Dimethyl sulphoxide (DMSO)	
Drabkin's reagent	
Glucose	
Glutaraldehyde	
Magnesium chloride ($MgCl_2$)	
Methanol ($\geq$ 99.6%, laboratory used)	
Nitric acid (HNO_3)	
Potassium chloride (KCl)	
Potassium ferricyanide ($K_3[Fe(CN_6)]$	British Drug House (Poole, UK)
Potassium nitrate (KNO_3)	
Saponin	
Sodium chloride (NaCl)	
Sodium nitrate ($NaNO_3$)	
Sodium hydroxide (NaOH)	
Sucrose	
Triton™ X-100	
Name	**Supplier**
Trichloroacetic acid (TCA)	
Urea	

2.4 Solutions

All solutions utilised had an osmolality of 290 ± 5 mOsm·kg^{-1} unless otherwise stated. The pH of MOPS-buffered solutions was adjusted utilising the temperature of coefficient of MOPS $\frac{\Delta pH}{\Delta T[°C]}$ of -0.011 by addition of NaOH, HNO_3 or Tris base (Good & Izawa, 1972).

2.4.1 RBC wash solution

To eliminate Cl^- and prevent activation of the dehydration pathway, buffy coat RBCs were washed with MOPS-buffered solution (MBS-NO_3 / N-MBS) *pH 7.4 at RT).

145 mM	$NaNO_3$
10 mM	MOPS
5 mM	Glucose

MOPS-buffered solution (Cl-MBS) for RBC incubation.

145 mM	NaCl
10 mM	MOPS
5 mM	Glucose

Bicarbonate-buffered solution (BBS) for RBC incubation.

121 mM	NaCl
24 mM	$NaHCO_3$
10 mM	MOPS
5 mM	Glucose

2.4.2 O_2 saturation solution

0.6% w/v $K_3Fe(CN)_6$

0.3% w/v saponin

2.4.3 RBC morphology

0.3% of glutaraldehyde in Cl-MBS, pH 7.4 at RT.

To examine medium composition effect on sickling (Chapter 4, section 4.2.6), 30% of methanol was utilised in Cl-MBS, BBS and plasma to fix RBCs, pH 7.4 at RT.

2.4.4 Transport pathway measurement

Stock solutions of bumetanide (10 mM), ouabain (10 mM) were made in Tris base and distilled water. When experiments were carried out utilising reagents dissolved in DMSO, controls also contained the same final percentage of solvent (< 0.5% final).

(a) Tonometer incubation for influx measurement

N-MBS, pH 7.4 at 37 °C.

(b) Incubation media for influx measurement

Cl-MBS, pH 7.4 / pH 7.0 / pH 6.5 at 37 °C	**BBS, pH 7.4 / pH 7.0 / pH 6.5 at 37 °C**	**plasma, pH 7.4 / pH 7.0 / pH 6.5 at 37°C**
145 mM NaCl	121 mM NaCl	Autologous plasma
10 mM MOPS	24 mM $NaHCO_3$	100 µM Ouabain
5 mM Glucose	10 mM MOPS	10 µM Bumetanide
100 µM Ouabain	5 mM Glucose	
10 µM Bumetanide	100 µM Ouabain	
	10 µM Bumetanide	

(c) Measurement of potassium chloride cotransport (KCC) after activation by physiological stimuli or pharmacological reagents

Desired physiological stimuli / pharmacological reagents in N-MBS + ouabain + bumetanide

Desired physiological stimuli / pharmacological reagents in Cl-MBS + ouabain + bumetanide

(d) $^{86}Rb^{+}$

$^{86}Rb^{+}$ (final activity around 0.05 $MBq.ml^{-1}$) in 150 mM KNO_3

(e) Mg-MBS wash solution, pH 7.4 at 4 °C

An ice-cold isotonic $MgCl_2$ solution (osmolality 290 $\pm$ 5 $mOsm \cdot kg^{-1}$) was utilised to eliminate unincorporated $^{86}Rb^{+}$

107 mM $MgCl_2$ 10 mM MOPS

2.5 Equipment

- Advanced Instruments Micro-Osmometer Model 3MO Plus, Advanced Instruments Inc., Fisher Scientific, Norwood, U.S.
- Balances: Sartorius, Basic BA61 and Sartorius MC210P, Bradford, MA, U.S.
- BD Vacutainer® test tubes, Becton Dickison, Plymouth, U.K.
- Centrifuge: type 541D, Eppendorf Ltd, U.K.
- Clark-type oxygen electrode and an oxygen meter CB1-D3, Hanstech, King's Lynn, U.K.
- Disposable cuvettes (1.5 ml, 12.5x12.5x45 mm), GMBH + CO KG, Wertheim Germany.
- Double beam UC spectrometer, CE5501, Cecil Instruments, Cambridge, U.K.
- Fisherbrand™ microhematocrit capillary tubes, Fisher Scientific U.K. Ltd., Loughborough, U.K.
- Fisherbrand™ Hemato-Seal™ Capillary Tube Sealant, Fisher Scientific U.K. Ltd., Loughborough, U.K.
- Flow cytometer BD Accuri™C6, BD Accuri C6 Software, Becton Dickinson, Biosciences, San Jose, Ca U.S.A.
- Gas mixing pump: H. Wösthoff Messtechnik GmbH, Bochum, Germany
- GraphPad Prism version 6.0c, San Diego, CA, U.S.
- Hettich Hematocrit 210 Centrifuge, Andreas Hettich GmbH & Co., Tuttlingen Germany
- Kutofix c tonometers, Eschweiler, Kiel, Germany.
- Memmert oven, Memmert GmbH + Co. KG, Schwabach, Germany
- Leica DM6000B microscope, phase contrast, 20x/0.70 PH2 (HC PLAN APO) ∞/0.17/C, equipped with a digital camera AF600DFC (magnification x1.6), Leica Microsystems Wetzlar GmbH, Milton Keynes, U.K.

- Microsoft Office 2017 Excel version15.33, Washington, U.S.
- Micro haematocrit centrifuge haematospin 1400, Hawksley, Lancing, U.K.
- N_2 cylinder, Surrey, England, U.K.
- O_2 cylinder, Surrey, England, U.K.
- pH meter: SevenEasy and combination liquid filled electrodes, Mettler-Toledo AG, Analytical, Sonnenbergstrasse, Switzerland.
- Tri-carb liquid scintillation counter 2800TR, Perkin Elmer, Waltham, U.S.
- Water Bath: Grant, Grant Instruments (Cambridge) Ltd, Shepreth, Cambridgeshire, U.K. and VWR Techne, Bibby Scientific Ltd, Stone Staffordshire, U.K.

3 Methods

3.1 Sample collection and handling

All sheep RBC samples were collected from LK sheep (~10 mM) (Evans, 1954), which have a high capacity of KCl cotransport. Before carrying out the experiment, the level of extracellular [K^+] in LK sheep plasma were adjusted to 7.5 mM. Hence, it was important to measure intracellular [K^+] and plasma [K^+]. The [K^+] in sheep RBCs and plasma were measured by The Queen's Veterinary School Hospital Central Diagnostic Services, and sheep plasma [K^+] was then adjusted to 7.5 mM by addition of an appropriate volume of 100 mM KNO_3.

With regards to human HbSS RBCs, which were routinely collected by Professor David C. Rees from King's College Hospital, their extracellular [K^+] were adjusted to the same value (7.5 mM). The [K^+] in HbAA / HbSS RBCs and plasma were measured by The Queen's Veterinary School Hospital Central Diagnostic Services, and HbAA / HbSS RBC's plasma [K^+] was then adjusted to 7.5 mM by addition of an appropriate volume of 100 mM KNO_3.

3.2 Testing the linearity of the gas mixing pump

As a gas mixing pump was extensively utilised in all further experiments, it was important to examine its accuracy. This was carried out by measuring O_2 tensions, utilising a Clark-type oxygen electrode linked with an oxygen meter CB1-D3. The gas mixing pump was set from 100% to 0% air. The gas mixture went through water into a Clark-type oxygen electrode chamber (Figure 3.1).

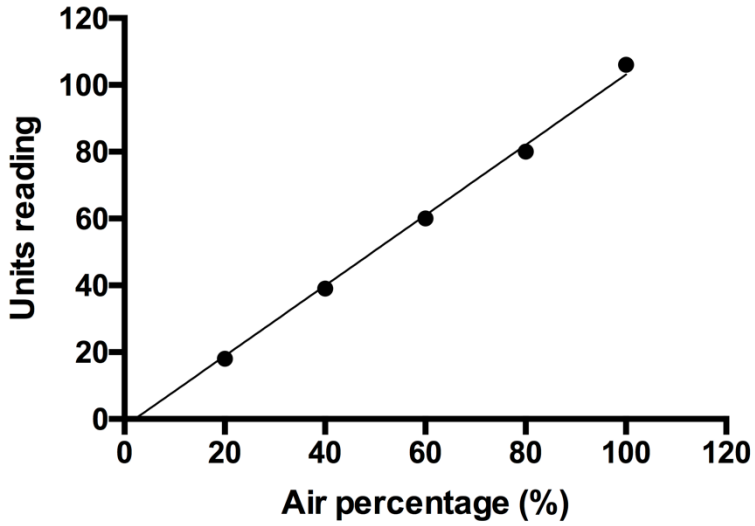

Figure 3.1 Linearity test of the H. Wösthoff Messtechnik gas mixer pump. The gas mixer was connected to the Clark-type electrode coupled to an oxygen meter CB1-D3 and then equilibrated with gas mixture for 10 min. Symbols represent the O_2 tensions under different percentages of air, means ± S.E.M., n= 3.

3.3 Haematocrit determination

To determine the volumetric proportion of RBCs in the suspensions, the measurement was determined by microhaematocrit centrifuge. Fisherbrand™ microhaematocrit capillary tubes were filled with RBC suspensions (by capillary) and centrifuged for 5 min at 13,000 *x g* utilising a Hawksley Haematospin 1400 Centrifuge. The ratio of RBC pellet to whole blood was determined utilising microhaemocrit reader.

3.4 Measurement of water content of RBCs

RBC water content was determined in triplicate and presented as ml water per g dry cell solids ($ml.g^{-1}$ d.c.s.). RBC samples were firstly washed in N-MBS (pH 7.4 at room temperature) and packed by 600 *x g* centrifugations. Then, the supernatant was removed. Samples were placed in EP tubes. Each EP tube contained 90 µl – 120 µl RBC suspension (together with various drugs, as required, or solvent in controls). To induce the influx or efflux of solute from sheep RBCs, the EP tubes were then pre-incubated at 37 °C for 45 min. Where necessary, RBCs sample were transferred to tonometer and incubated for 60 min at the required O_2 tensions. To stop the solute fluxes, RBCs samples were aliquoted into three pre-chilled EP tubes kept on ice and subsequent procedures were all carried out at 4 °C. RBC suspensions were then transferred into capillary tubes and centrifuged for 15 min at 11,000 *x g*, to obtain

tightly packed RBC pellets. After that, empty foil squares were dried and weighed. The pelleted RBCs from the capillary tubes were expressed onto the foil squares and their wet weight were measured carefully. RBC pellets were then dried at a temperature of 95 °C – 100 °C, for more than 18 hours, after which dry weights were taken. The water content in ml per g dry cell solids ($ml.g^{-1}$ d.c.s) was calculated by the following equations: wet weight = (wet+foil) - foil; water/cell = (wet+foil) – (dry+foil); dry weight = (dry+foil) – foil; $ml.g^{-1}$ d.c.s. = (water/cell)/ (dry weight).

3.5 Measurement of intracellular pH

The RBC suspensions were placed into a tonometer and equilibrated with required nitrogen/air/carbon dioxide gas mixture, which was saturated with humidified gas mixtures at 37 °C. RBC suspensions were incubated under the desired gas mixture composition, in a gently rotating tonometer for 15 min. To maintain equilibration with gases, RBC samples were then transferred into small EP tubes and span through DBP (dibutyl phthalate) oil at 600 *x g* for 3 minutes. The packed RBCs were frozen and thawed twice to ensure complete haemolysis. The pH values of the lysed solution were measured at 37 °C using a pH meter.

3.6 Oxygen saturation curve

Oxygen saturation curves (OSC) were determined at 37 °C. This method was achieved by placing RBC suspensions (50% Hct) in an Eschweiler tonometer in a water bath at 37 °C. The tonometer was connected to the gas mixer and samples were equilibrated at the required O_2 tension (0% or 100% air).

An appropriate volume of 10 µl RBC suspensions were added to a saponin (0.3%) / ferricyanide (0.6%) solution in the Clark-type electrode using a Hamilton syringe. The saponin / ferricyanide solution was used to lyse the RBCs and displaced oxygen from haemoglobin. The initial and final readings were then recorded. The difference in readings made with RBCs equilibrated with 0 mmHg O_2 and fully oxygenated (at 150 mmHg O_2) were used to obtain a volume for 100 % O_2 saturation. O_2 saturations were then measured at various time points (time points: 0 min, 6 min, 9 min, 12 min, 15 min) to ascertain how quickly O_2 equilibrations were achieved.

Herein, the Hb oxygen saturation in blood samples was investigated by determining two oxygen equilibrium curves at the same pH. To fully oxygenate or deoxygenate the sheep RBC samples, the samples (50% Hct) were equilibrated for up to 15 min at the required O_2 tension before measuring Hb O_2 saturation by utilising a Clark-type oxygen electrode and an oxygen meter CB1-D3. In the first experiment, the Hb O_2 saturation was measured during re-oxygenation changing O_2 tensions from 0% air (PO_2= 0 mmHg) to 100% air (PO_2= 150 mmHg). Meanwhile, for comparison, the Hb deoxygenation rate RBCs from 100% air (PO_2= 150 mmHg) to 0% air (PO_2= 0 mmHg) was measured as well.

In both cases, 50% change in O_2 saturation was achieved after about 8 min. Re-oxygenation was completed by 12 min, whilst de-oxygenation took relatively longer and was achieved after about 15 min. In subsequent experiments, equilibration with gases was taken over 15 min.

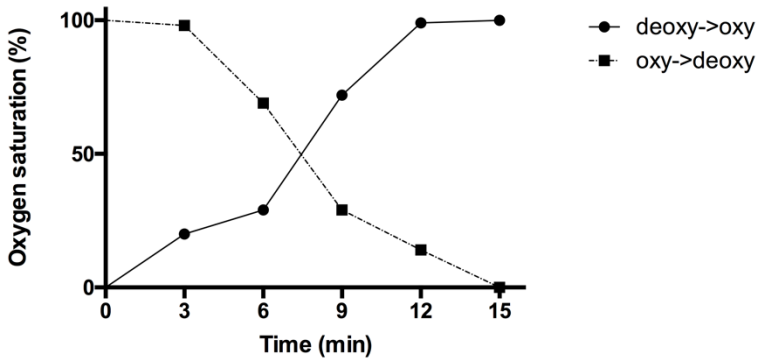

Figure 3.2 Time course of the change in Hb oxygen saturation of RBCs. The solid line represents sheep RBCs re-oxygenated from 0% air to 100% air. The dashed line represents RBCs de-oxygenated from 100% air to 0% air. The experiment utilised sheep RBCs but similar results were obtained with human RBCs.

3.7 Measurement of RBC sickling

RBCs were incubated in tonometers for 15 min at 37 °C at each oxygen tension and then fixed in N-MBS (0.2% haematocrit) containing 0.3% glutaraldehyde which would maintain RBC shape for several weeks (Milligan *et al.*, 2013). The shape of RBC was studied under a Leica DM6000B light microscope. Elongated RBCs with egg-shape or pencil forms with spiky turns were counted as sickled. At least 500 RBCs were counted, and results given as a percentage of biconcave and elongated (sickled) RBCs.

3.8 Measurement of PS exposure in HbSS RBCs

3.8.1 Lactadherin-FITC (fluorescein isothiocyanate) titration

Lactadherin is a 45-kda glycoprotein milk fat globule-EGF factor 8, which is generated by macrophages and has an ability to bind to PS on apoptotic cells, promoting their clearance by macrophages (Hanayama *et al.*, 2002; Albanyan *et al.*, 2009; Zhou *et al.*, 2009). Another protein which is called Annexin V has been utilised to detect PS exposure in HbSS RBCs (Dasgupta *et al.*, 2006; Albanyan *et al.*, 2009). According to Albanyan *el al.*'s and Zhou *et al*.'s study, it is Ca^{2+} dependent and requires a higher threshold of PS exposure (2% - 8%) in HbSS RBCs, which makes it less useful for the current set of experiments (Albanyan *et al.*, 2009; Zhou *et al.*, 2009). Comparing to annexin V, lactadherin has a higher sensitivity to detect PS exposure at lower level of PS exposure (0.5%) and binds independent of PE membrane and Ca^{2+} (Shi *et al.*, 2006; Waehrens *et al.*, 2009; Zhou *et al.*, 2009). Based on previous research, the optimum condition for labelling exposed PS on RBCs is to incubate at room temperature for 10 min (Weiss *et al.*, 2011). In this study, each batch of LA-FITC titrated was to find out the minimal concentration required. RBCs were incubated in HK saline (100 μM $[Ca^{2+}]_o$) with 6 μM calcium ionophore Br-A23187 (0.5% Hct) for 30 min at 37 °C. After that, 5 μl (about 10^5 RBCs) of the cell suspension was added to tubes containing 250 μl of HK saline (100 μM $[Ca^{2+}]_o$) with different concentrations of LA-FITC (0 - 32 nM) in duplicates, for fluorescence labelling of exposed PS. Labelling of PS was carried out at room temperature for 10 min in the dark. Detection of PS exposure increased with LA-FITC concentration (Figure 3.3). Although the cells were not fully saturated at 16 nM, it yielded a high enough detection signal for PS exposure with minimal effective concentration of LA-FITC. Hence, this concentration was utilised in all further experiments.

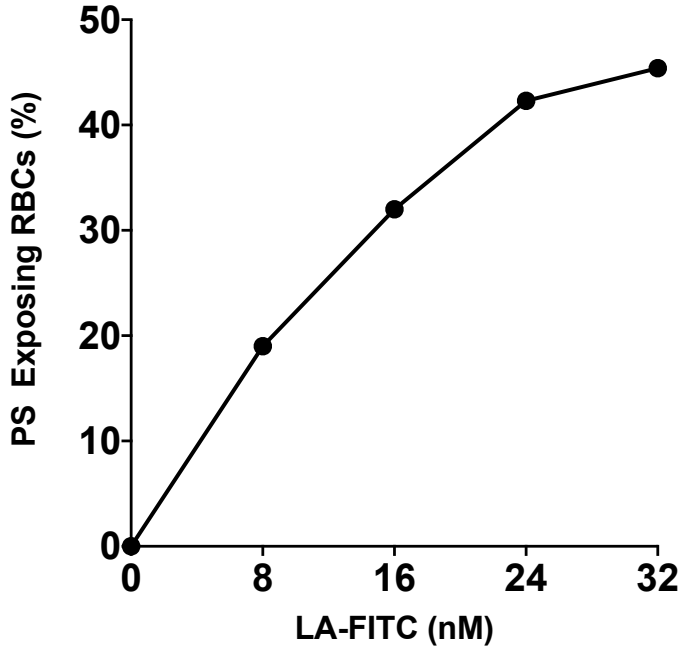

Figure 3.3 Optimization of LA-FITC binding to PS exposed on RBCs from SCA patients. After 30 min incubation at 37 °C with Br-A23187 (6 µM, 0.5 % Hct), RBCs were harvested and added to lactadherin-binding saline with LA-FITC (100 µM $[Ca^{2+}]_o$ HK saline). Lactadherin-labelling of PS exposing RBCs was carried out for 10 min in the dark at room temperature after which RBCs were washed once (600 *x g* **g** for 3 min, followed by short spin; 16,000 *x g* for 10 s) and kept on ice until FACS analysis. Symbols represent a single experiment representative of 2 others.

3.8.2 Calcium loading of RBCs with ionophore

PS exposure was stimulated in RBCs by utilising a Ca^{2+} ionophore. After the proper washes in HK saline, to remove contaminant Ca^{2+}, RBCs were washed once into LK or HK saline containing 2 mM EGTA. To alter intracellular calcium concentration ($[Ca^{2+}]_i$), RBCs (0.5 % Hct) were then incubated with Br-A23187 (dissolved in DMSO; final concentration of DMSO was 0.5%) and different $[Ca^{2+}]_o$s clamped with 2 mM EGTA in LK or HK saline. To inhibit the plasma membrane Ca^{2+} pump (PMCA) and flippase during Ca^{2+} loading, vanadate (1 mM in aqueous solution) was also added to RBC solution. Ca^{2+} entry and stimulation of the Ca^{2+} pump would otherwise rapidly deplete ATP and could lead to secondary effects. Br-A23187 is non-fluorescent with an apparent transport selectivity for Ca^{2+} over Mg^{2+} (Debono *et al.*, 1981). This transportation is electroneutral via diffusion through the membrane leading to an exchange of one Ca^{2+} for two H^+, which slowly increases intracellular (about 0.1 units/10 min) (Erdahl *et al.*, 1995). Also, the extent of Ca^{2+} transport is dependent on the concentrations of Br-A23187. $[Ca^{2+}]_i$ is fixed according to free $[Ca^{2+}]_o$ and the Donnan equilibrium ratio (r) $[Ca^{2+}]_i = [Ca^{2+}]_o \times r^2$, where r^2 equals 2.05 for the

membrane of RBCs from SCA patients (Flatman & Lew, 1977; Muzyamba *et al.*, 2006). Results showed that the flux of Ca^{2+} with Br-A23187 stimulated maximal PS exposure in RBCs from SCA patients by 20 min incubation, therefore, incubation for all the experiments on Br-A23187 was carried out at 37 °C for 30 min (Figure 3.4a).

The optimal concentration of Br-A23187 required to clamp $[Ca^{2+}]_i$ reliably for PS exposure was nominally 2 - 6 µM (Figure 3.4). The $[Mg^{2+}]_o$ in these experiments was usually 0.15 mM to keep $[Mg^{2+}]_i$ at physiological levels. Due to the altered activity of different batches of ionophore Br-A23187 varied, each was titrated to set up the minimal concentration required to clamp $[Ca^{2+}]_i$. RBC suspensions were oxygenated at all times and incubated for 30 min in Eppendorf tubes (1.5 ml) in plastic racks which were immersed in water bath at 37 °C. Aliquots (5 µl, 10^5 RBCs) were labelled with LA- FITC and analysed by FACS (Figure 3.4b).

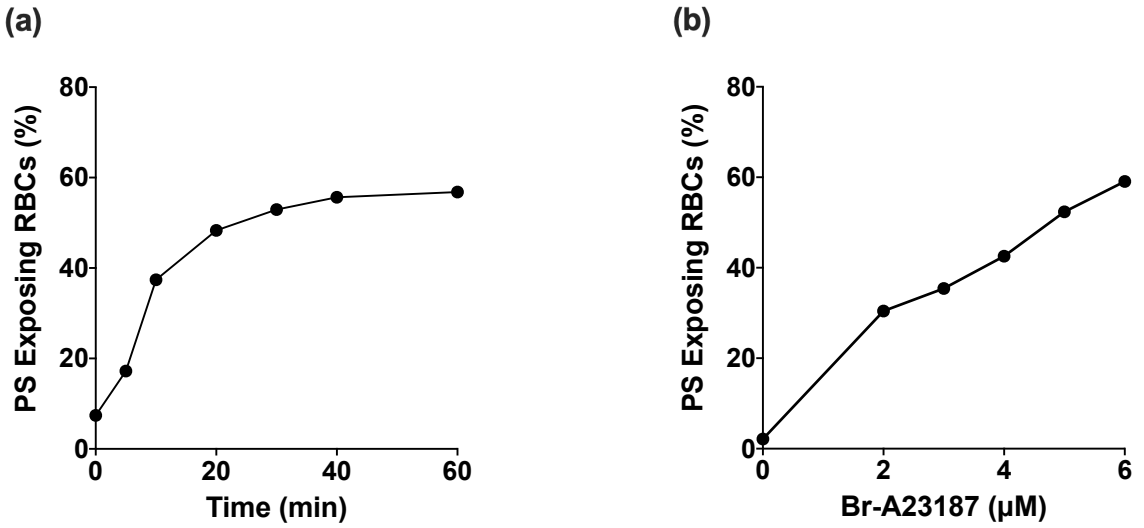

Figure 3.4 Optimization of Ca^{2+} ionophore Br-A23187 in RBCs from SCA patients. RBCs (0.5% Hct) were first treated with vanadate (1 mM) to inhibit the plasma membrane Ca2+ pump (PMCA) and flippase before incubation for 30 min at 37 oC with Br-A23187. (a) Br-A23187 (6 µM) incubation time course in HK saline containing 100 µM $[Ca^{2+}]_o$, before labelling with LA-FITC (b) RBC suspension with Br-A23187 (nominally 0 - 6 µM) in HK saline containing 100 µM $[Ca^{2+}]_o$. Symbols represent a single experiment representative of 2 others.

3.9 Time course of the pH change in BBS/plasma at different partial pressures of CO_2

The pH value of BBS and plasma was adjusted by altering the partial pressure of CO_2. To explore the pH value change in BBS and plasma, BBS/plasma was gassed with desired $O_2/CO_2/N_2$ gas composition at 37°C for up to 6 min. The pH value change in BBS/plasma was measured every minute. Results confirmed that the pH value of each buffer system gassed with desired gas composition became steady within 4 min. The pH values of BBS and plasma were calculated by utilising Henderson-Hasselbalch equation: $pH = pKa + \lg(\frac{[HCO3^-]}{0.03*PCO_2})$, ("pKa = 6.1", a negative logarithm of the acid dissociation constant for H_2CO_3, and "0.03*PCO_2" was $[H_2CO_3]$).

Figure 3.5 shows a plot of the pH value change in BBS during a 6 min incubation with different partial pressures of CO_2 (PCO_2= 7.13 mmHg, PCO_2= 43 mmHg, PCO_2= 100 mmHg, PCO_2= 321 mmHg). To obtain pH 7.4, pH 7.0 or pH 6.5 BBS, BBS needed to be incubated at 37°C and equilibration with appropriate partial pressures of CO_2 for at least 4 min.

The pH change in plasma equilibrated at different PCO_2(s) showed similar curves in Figure 3.6.

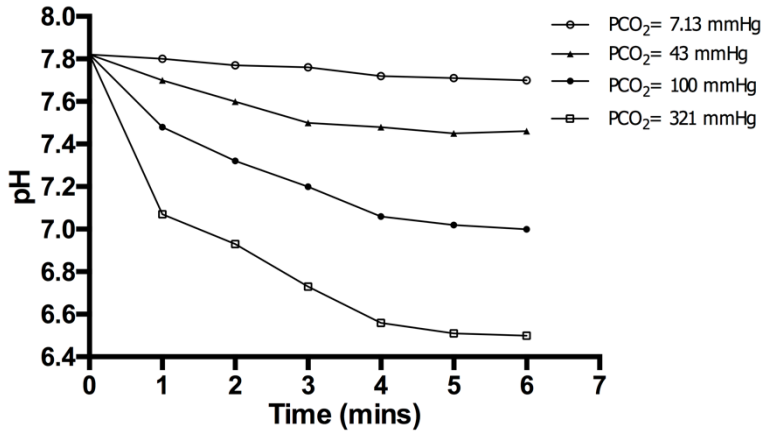

Figure 3.5 Time course of the pH change in BBS altered by different partial pressures of CO_2. BBS was gassed with desired gas composition and equilibrated in tonometer at 37°C for 6 minutes. (○), PCO_2= 7.13 mmHg incubation; (▲), PCO_2= 43 mmHg incubation; (●), PCO_2= 100 mmHg incubation; (□), PCO_2= 321 mmHg incubation.

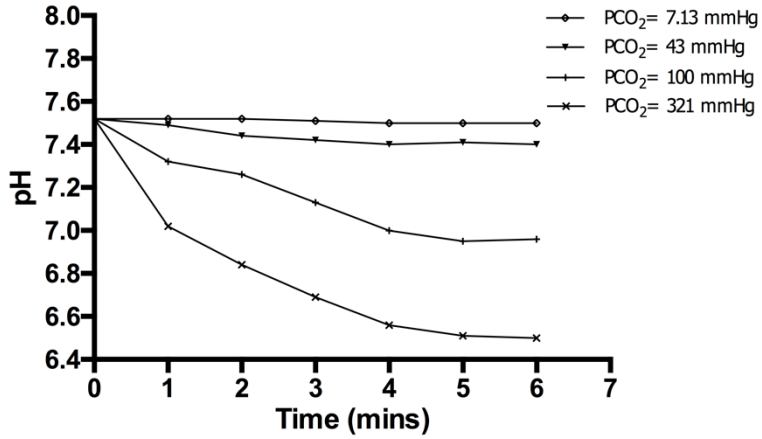

Figure 3.6 Time course of the pH change in plasma altered by different partial pressures of CO_2. Plasma was gassed with desired gas composition and equilibrated in tonometer at 37°C for 6 minutes. (◇), PCO_2= 7.13 mmHg incubation; (▼), PCO_2= 43 mmHg incubation; (+), PCO_2= 100 mmHg incubation; (×), PCO_2= 321 mmHg incubation.

3.10 Control experiment – intracellular RBC [K^+] and extracellular plasma [K^+] in LK sheep and human HbSS RBCs

Before carrying out the sheep flux experiments, the intracellular RBC [K^+] and extracellular plasma [K^+] of LK sheep, and that of plasma in human HbSS samples were measured (Table 3.1). Table 3.1 refers just to sheep samples but the same procedure was utilised to adjust the extracellular [K^+] in HbSS plasma.

At the beginning of each experiment, the extracellular plasma [K^+] was adjusted to 7.5 mM utilising a stock solution of 100 mM KNO_3 as any variation in [K^+] would affect K^+ influx. Table 6.1 shows the results of intracellular RBC [K^+] and extracellular plasma [K^+] in different LK sheep. The mean value of intracellular RBC [K^+] was 12.8 ± 1.6 mM (mean ± S.E.M, n=11). The mean value of extracellular plasma [K^+] was 4.2 ± 0.2 mM (mean ± S.E.M, n=11). The results confirmed that all sheep utilised in this project were LK, in which the intracellular RBC [K^+] was about 10 mM (Evans, 1954).

Table 3.1 The intracellular RBC [K^+] and extracellular plasma [K^+] of sheep blood used in various experiments.

Sheep	Intracellular [K^+] (mM)	Extracellular [K^+] (mM)
1	15	4.4
2	26	4.3
3	7	4.4
4	13	4.1
5	13	4.5
6	11	4.1
7	8	4.1
8	7	4.8
9	15	5.1
10	16	4.2
11	10	2.6
Mean	12.8	4.2
Mean ± S.E.M	12.8 ± 1.6	4.2 ± 0.2

3.11 Control experiment – water content of RBCs

KCC activity can be altered by cell volume, therefore, it was important to check whether the RBC volume was changed by incubation in Cl-MBS, BBS and autologous plasma. Table 3.2 presents the water content in different sheep RBCs all at pH 7.4. Each measurement was carried out in triplicate. The mean value of RBC water content incubation with Cl-MBS was 1.97 ± 0.04 $mg \cdot g^{-1}$ d.c.s (mean ± S.E.M, n=11). With regards to sheep RBCs incubation with BBS, the mean value of RBC water content was 1.91 ± 0.04 $mg \cdot g^{-1}$ d.c.s (mean ± S.E.M, n=11). With regards to the sheep RBCs incubation with plasma, the mean value of RBC water content was 1.95 ± 0.05 $mg \cdot g^{-1}$ d.c.s (mean ± S.E.M, n=11). Though the mean ± S.E.M value of water content in RBC in BBS was slightly lower than in Cl-MBS or in plasma, the results confirmed that incubation media did not have a significant effect on RBC volume. Similar results were obtained with human HbSS RBCs

Table 3.2 The water content of RBCs after incubation with Cl-MBS, BBS and plasma at pH 7.4. Each determination was carried out in triplicate. No significant differences (n.s.) in volume were found after incubating in either Cl-MBS, BBS or plasma by Student's t test.

Sheep	The water content of RBCs (Cl-MBS incubation) ($mg \cdot g^{-1}$ d.c.s)	The water content of RBCs (BBS incubation) ($mg \cdot g^{-1}$ d.c.s)	The water content of RBCs (plasma incubation) ($mg \cdot g^{-1}$ d.c.s)
1	1.79	1.74	1.73
2	2.25	2.07	2.18
3	2.01	1.95	1.97
4	1.95	2.11	2.07
5	1.82	1.76	1.78
6	2.01	1.95	1.90
7	1.82	1.72	1.76
8	2.05	1.93	1.98
9	1.96	1.84	1.89
10	2.01	1.95	2.08
11	2.03	2.01	2.15
Mean	1.97	1.91	1.95
Mean ± S.E.M.	1.97 ± 0.04 (n.s.)	1.91 ± 0.04 (n.s.)	1.95 ± 0.05 (n.s.)

3.12 Measurement of the K^+ influx

The permeability of several K^+ pathways was measured by utilising radioisotope $^{86}Rb^+$, which is a known K^+ congener. $^{86}Rb^+$ (final concentration (0.02-0.05 MBq/ml) was added to all tubes at a final [K^+] of 7.5 mM. To remove unincorporated radioisotope $^{86}Rb^+$ in each tube and terminate fluxes, RBC suspensions were added into EP tubes, which contained ice-chilled $MgCl_2$ saline. Then, RBC suspensions were centrifuged at 600 *x g* for 10 seconds. The supernatants were then removed, and another aliquot of wash solution added. This washing cycle was repeated four more times. After the final wash, 0.5 ml of 0.01% Triton X-100 was added to lyse packed RBCs. To precipitate protein, 0.5 ml 5% trichloroacetic acid was added to EP tubes. The EP tubes were then centrifuged at 13,000 rpm for 5 minutes. The $^{86}Rb^+$-containing supernatant was

analysed by Čerenkov radiation in a liquid scintillation counter. The K^+ influxes were calculated as micromoles K^+ per litre per hour by utilising the following equation:

$$Influx\ [\mu mol.(l\ cells.h)^{-1}] = \frac{sample\ cpm - background\ cpm}{specific\ activity\ of\ ^{86}Rb^+} \times \frac{60}{flux\ time\ (\min)} \times \frac{1000}{flux\ volume\ (ml)} \times \frac{100\ (\%)}{flux\ Htc\ (\%)}$$

Herein, the term "cpm" represents counts per minute each tube.

Specific activity of $^{86}Rb^+$= total cpm counts of $^{86}Rb^+$ per tube divided by total µmol K^+ per tube.

3.13 Statistics

Data are presented either as individual results representative of at least 3 experiments on different individuals or as means ± S.E.M. in RBC samples from n different individuals. Statistical significance of paired variables was analysed by Student t-test or one-way ANOVA with Tukey (to compare paired values) or Dunnett's (to compare to a control value) by utilising Graphpad Prism software (La Jolla, CA, USA). Correlations were tested utilising Pearson correlation test. A value of $p < 0.05$ was considered significant; calculations were made utilising Microsoft Office Excel or Graphpad Prism software.

4 HbS polymerisation and sickling

4.0 Introduction

Erythrocyte sickling and haemolysis due to HbS polymerisation upon deoxygenation is a hallmark morbidity in those patients with sickle cell anaemia (SCA). Accordingly, inhibition of HbS polymerisation is a vital non-genetic therapeutic approach to ameliorate the sequelae of sickle cell HbS polymerisation such as vaso-occlusive crises (VOCs) and acute chest syndrome (Pauling *et al.*, 1949; Ingram, 1957; Marotta *et al.*, 1977). There are two major physiological characteristics which tend to induce HbS polymerisation and fibre formation: hypoxia and acidosis. Under a hypoxic environment, HbS molecules tend to aggregate, undergoing a homogeneous nucleation event by forming single fibres. This may be followed by a second polymerisation event, the heterogeneous nucleation, in which branches of new fibres are added to the surface of existing ones (Ferrone *et al.*, 1980; Eaton & Hofrichter, 1990). Moreover, in an acidotic environment, the low pH decreases the solubility of HbS which results in an increased driving force for HbS polymerisation, as observed in SCA patients (Ueda & Bookchin, 1984). In order to understand how key physiological factors affect HbSS RBC morphology, experiments were carried out to examine their effects on sickling in HbSS RBCs under three different oxygen tensions: 150, 30 and 0 mmHg; to investigate the effect of rate of deoxygenation on sickling of HbSS RBCs; effect of different pHs on sickling; effect of urea at levels found in the renal medulla (in presence of 300 mM NaCl) and the effect of anisotonicity (i.e. cell volume).

Over the past five decades, a number of anti-sickling drugs / therapeutics have been proposed including urea at high salt level (Mahmood, 1974), a dehydration manoeuvre (Brugnara, 2001) which has previously been suggested to prevent HbS fibres formation due to interference by urea with hydrophobic bonds responsible for the HbS polymerisation and by lowering the activity and concentration of intracellular HbS (Eaton, 2002). Moreover, five drugs were utilised to understand their pharmacological roles in sickling of HbSS RBCs, as they will be used in future chapters to examine their effect on one of the major dehydration pathways involved in pathogenesis, the KCl cotransporter or KCC (Chapters 6 and 7): staurosporine, a potent and non-selective

inhibitor of protein kinases which also induces cell apoptosis by an unknown mechanism; calyculin A, a potent phosphatase inhibitor for triggering red cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane (Almasry *et al.*, 2016); *N*-ethylmaleimide (NEM), a drug normally used for blocking cellular sulfhydryl groups in proteins and peptides; WNK463, the first orally bioavailable pan-WNK-kinases inhibitor for inhibiting with-no-lysine kinases (WNKs). Overall, a better understanding of their pharmacological effects on HbSS RBC morphology could serve to elucidate the pathophysiology of SCD, may inform potential therapeutic approaches and also act as important control experiments for later work examining their effects on KCC activity.

Whilst there is an extensive literature on HbSS sickling and conditions affecting HbS polymerisation, most experiments in this thesis are carried out using special Eschweiler tonometers to equilibrate RBC samples under the requisite conditions of oxygen, carbon dioxide and pH. It was therefore important to determine carefully the effects of these different conditions on sickling, using this particular apparatus, as they are important control experiments which are necessary for the interpretation of later experiments in which phosphatidylserine (PS) exposure and KCC activity are examined in detail.

4.1 Results

4.2 Effect of physiological stimuli on the morphology of HbSS RBCs

4.2.1 Effect of different oxygen tensions (150, 30 and 0 mmHg) on sickling

Oxygen tension (PO_2) is one of the most important physiological parameters which affect sickling of HbSS RBCs. The polymerisation of sickle haemoglobin is an equilibrium between insoluble HbS crystals and dissolved HbS in solution. The kinetic features of HbS polymers formation underlie critical determinants of sickle RBCs morphology. Meanwhile, robust evidence suggest that sickle cell suspensions and HbS in solution are strongly dependent on PO_2 (Winslow, 2006). In this study, HbSS RBCs sickling conditions were investigated under three oxygen tensions: 150, 30 and 0 mmHg.

At an oxygen tension of 150 mmHg, only biconcave HbSS RBCs were observed in RBCs solution, which implied HbSS RBCs were fully oxygenated (Figure 4.1). However, the proportion of elongated HbSS RBCs rose to approximately 60% and that of biconcave HbSS RBCs proportion decreased to about 40% when RBC aliquots were exposed to an oxygen tension of 30 mmHg. When HbSS RBCs were incubated at 0 mmHg oxygen tension, the proportion of elongated RBCs significantly increased to 80% and that of biconcave HbSS RBCs proportion fell to 20%, which implied that HbSS RBCs were significantly deoxygenated. Moreover, the proportion of elongated RBCs in 0 mmHg group was 25% ($p < 0.05$) higher than that in 30 mmHg group.

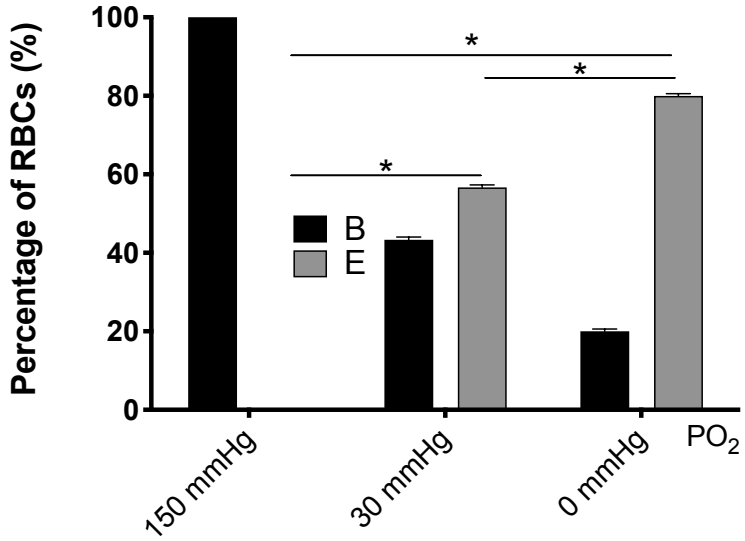

Figure 4.1 Effect of different oxygen tensions on sickling of HbSS RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers for 15 min at the required oxygen tensions (150 mmHg, 30 mmHg or 0 mmHg) in Cl-MBS. Experimental pH was pH 7.4 at 37 °C. Glutaraldehyde (0.3% as final concentration) was then added into the RBC aliquots which were then incubated for 10 min at the same oxygen tension before cell counting under light microscopy. In this figure, "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

4.2.2 Effect of rate of deoxygenation on sickling

Rotation of the tonometer is an important way to evenly mix the RBC suspensions and ensure that they are fully equilibrated at the desired oxygen tensions. In many SCA patients, individuals are suffering from vaso-occlusive crises, which in turn induce tissue ischemia and infarction. The consequences of vaso-occlusion are mainly caused by exposure of RBCs from high-velocity and high-oxygen levels, for example in cerebral arteries, to a sudden change to low-velocity and low-oxygen postcapillary venules. Rate of deoxygenation is also known to affect the balance between homogeneous and heterogeneous nucleation and hence RBC morphology, which may impact upon cell behaviour. Therefore, it is important to understand the effect of rate of deoxygenation on RBC morphology from SCD patients. In this work therefore, the effect of different rates of deoxygenation (adjusted by controlling rotation speed of tonometer) on sickling were compared.

HbSS RBC suspensions (1% Hct, 0.5 ml) were first incubated in Cl-MBS for 15 min in Eschweiler tonometers at 37 °C, which were flushed with warm and humidified N_2 or air at 5-10 bubbles.s^{-1} with different rotation speeds. To fix the RBC morphology, glutaraldehyde (0.3% final concentration) was then added to the RBC suspensions for

10 min. The RBC suspensions were taken from the tonometers and transferred to Eppendorf tubes for cell counting.

As shown in Figure 4.2, results indicated that no elongated RBCs were found in RBC suspensions at an oxygen tension of 150 mmHg, regardless of the rotation speed of the tonometers. However, at an oxygen tension of 0 mmHg, at high tonometer rotation speed (180 rpm), the number of elongated RBCs increased to 80% and the number of biconcave RBCs fell to 20%. While at a medium tonometer rotation speed of 100 rpm and at the slowest rotation speed of 80 rpm, the number of elongated RBCs increased to 75% and 60% at an oxygen tension of 0 mmHg. The number of biconcave RBCs fell to 25% at the medium rotation speed (100 rpm) and 35% at the slowest rotation speed (80 rpm) RBCs, all at an oxygen tension of 0 mmHg.

Comparing the RBC morphology at 180 rpm (PO_2=0 mmHg) with that at 100 rpm tonometer (PO_2=0 mmHg), elongated RBC number was 6% ($p < 0.05$) higher than that at the 100 rpm rotation speed, whilst there were 33% more elongated RBC number at 180 rpm ($p < 0.05$) compared with those at 80 rpm, and 25% higher when comparing the elongated RBCs level at 100 rpm with that at 80 rpm ($p < 0.05$).

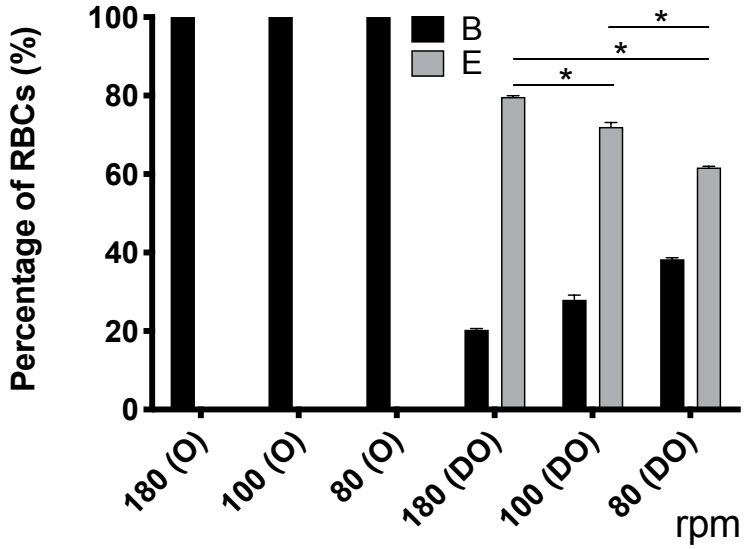

Figure 4.2 Effect of rate of deoxygenation on sickling of HbSS RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The rate of deoxygenation was controlled by adjusting the rotation speed of Eschweiler tonometers. In all of the experiments, the tonometers were flushed with the same gas bubble speed (5-10 bubbles.s^{-1}). The aliquots were fixed as described in the legend to Figure 4.1. Herein, "rpm" represents "revolutions per minute". "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

4.2.3 Effect of extracellular pH on sickling

Extracellular pH is another key physiological parameter which determines the HbSS RBC morphology. The normal biconcave shape and deformability of human RBCs are important biological functions which allow RBCs to pass through small capillaries and transport oxygen and carbon dioxide throughout the microcirculation. In small blood vessels, RBCs alter their shape to squeeze through narrow parts of the circulation, and this ability is dependent on the non-covalent cytoskeleton of spectrin tetramers together with their attachments to the plasma membrane. However, in some SCA patients, sickle RBCs lose the ability to reverse their elastic deformation following passage through the blood microcirculation because of low surface to volume ratio and disruption to the cytoskeleton, leading to severe complications such as pulmonary hypertension (Pham *et al.*, 2000; Diez-Silva *et al.*, 2010). The renal medulla represents an area which poses particular challenges to HbSS RBCs, being both hypoxic and acidotic, which can induce morphology changes in HbSS RBCs, causing decreased medullary blood flow, microinfarct and renal papillary necrosis (Pham *et al.*, 2000;

Diez-Silva *et al.*, 2010). In this context, it is noticeable that around a third of SCA patients lack a functional renal medulla and the ability to concentrate their urine.

The following experiment (Figure 4.3) was designed to investigate the effect of pH across a wide range from pH 6 to pH 8 on sickling of HbSS RBCs. Sickling markedly increased in pH 7.4, pH 7.0 and pH 6.5, from 8% ($p < 0.05$) to 38% ($p < 0.05$) to 38% ($p < 0.05$), respectively, compared to that at pH 8 under 0 mmHg PO_2 environment. Sickling also significantly increased at pH 7.0 and at pH 6.5, which increased from 14% ($p < 0.05$) and 13% ($p < 0.05$), compared to the same number at pH 7.4. No significant change in sickling was observed as pH was decreased from pH 7.0 to pH 6.5 under a 0 mmHg PO_2 environment.

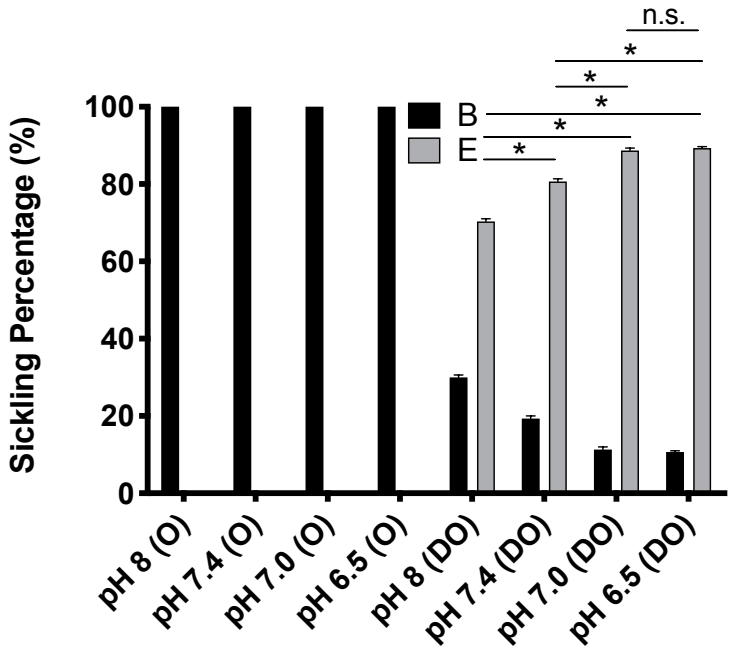

Figure 4.3 Effect of extracellular pH on sickling of HbSS RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in Cl-MBS (different pHs) for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

4.2.4 Effect of urea at levels found in the renal medulla (600 mM) in combination with hypertonic concentrations of NaCl on sickling

The sickling of HbSS RBCs was compared in the absence and presence of 600 mM urea in combination with 300 mM NaCl in Cl-MBS at oxygen tensions of 150 mmHg and 0 mmHg. These condition mimic those found physiologically in a healthy renal medulla during maximal antidiuresis. In Cl-MBS containing 600 mM urea and a total of 300 mM NaCl (PO_2 = 150 mmHg), sickling percentage was 62.5% ($p < 0.05$) lower than that in the absence of urea and high NaCl at an oxygen tension of 0 mmHg.

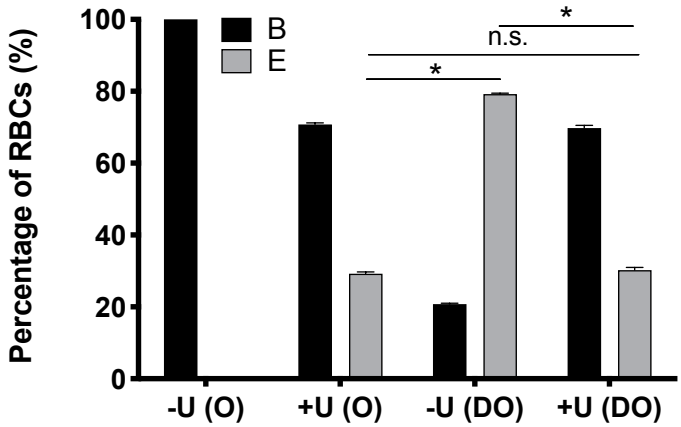

Figure 4.4 Effect of urea at concentrations found in the renal medulla (600 mM) in combination with hypertonic NaCl (300 mM NaCl). HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in Cl-MBS for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "-U" represents HbSS RBCs incubated with Cl-MBS in the absence of urea and 300 mM NaCl; "+U" represents HbSS RBCs incubated with Cl-MBS in the presence of urea and 300 mM NaCl. "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=4), n.s. indicates "not significant" by Student's t test.

4.2.5 Effect of hypotonic swelling on sickling

In this experiment, the effect of hypotonic swelling on sickling of RBCs was investigated. In all cases, osmolality, pH, extracellular [K^+] and O_2 tension were all carefully controlled. The osmolality of the basic Cl-MBS was altered by addition of water by up to 30%, either reducing it or raising it. Under these conditions, RBC water content was found to change by about 25% (Carden *et al.*, 2017). In Figure 4.5, the sickling percentage in RBCs swollen by 10%, 20% and 30%, decreased by 62% ($p < 0.05$), by 87% ($p < 0.05$) and by 97% ($p < 0.05$), compared to those RBCs at normal cell volume, respectively. Also, the sickling percentage in 20% swollen RBCs and 30%

swollen RBCs were 67% ($p < 0.05$) and 93% ($p < 0.05$) less than those which was 10% swollen RBCs at an oxygen tension of 0 mmHg. Moreover, sickling percentage in 30% swollen RBCs was 80% ($p < 0.05$) less than that in those swollen by 20%.

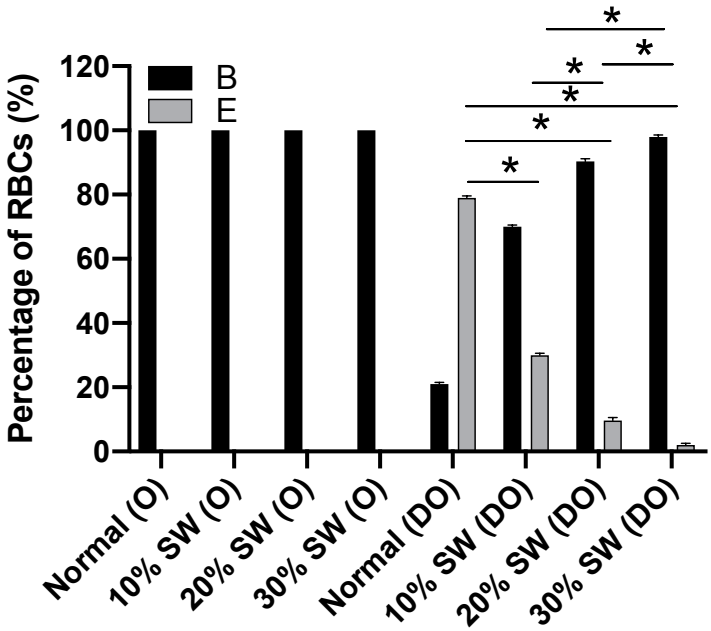

Figure 4.5 Effect of hypotonic swelling (i.e. cell volume) on sickling of RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in Cl-MBS for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. RBC volume was altered anisotonically through addition of water (so that the incubati0on media were made hypotonic by 10%, 20% and 30%) in Cl-MBS. The aliquots were fixed as described in the legend to Figure 4.1. "SW" represents HbSS RBCs were placed in Cl-MBS with addition of water and thereby swollen by 10%, 20% and 30%. "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=4).

4.2.6 Effect of buffer (Cl-MBS, BBS and autologous plasma) on sickling

RBC morphology was examined in Cl-MBS and compared to that in cells incubated in bicarbonate-buffered saline (BBS) and autologous plasma. Due to the fact that protein crosslinking on exposure to glutaraldehyde cause plasma to form a gel-like mixture, which caused RBC haemolysis and prevented analysis of RBC morphology, an alternative method of fixing the cells to avoid this problem was first established. It was found that methanol represented a good fixative which did not react in a deleterious way with plasma proteins.

In this case, RBCs were incubated with chilled and different methanol concentrations (using a stock of ≥ 99.6%, laboratory grade) for 10 min after carrying out the experiment. In order to fix aliquots in Cl-MBS, BBS and autologous plasma, control

experiments established the concentration and incubation time required to fix RBCs using methanol supplemented to Cl-MBS. Figure 4.6.1 (a) shows the survival rate of RBCs after 10 min incubation with different concentrations of methanol in Cl-MBS. Survival rate was found to be nearly 100% after incubation with 0% - 30% methanol (v/v%) in Cl-MBS at O_2 tensions of 150 mmHg and 0 mmHg. When the methanol / Cl-MBS volume ratio was increased to 40% (v/v%), RBCs started to undergo haemolysis and survival rates fell to less than 10%. All RBCs underwent haemolysis at a methanol concentration of 50% (v/v%), at O_2 tensions of 150 mmHg and 0 mmHg. In Figure 4.6.1 (b), sickling percentage was highest at 20% when the RBCs were fixed with 30% ratio of methanol at O_2 tensions of 0 mmHg compared to those fixed with 10% methanol (sickling percentage: 5%) and 20% methanol (sickling percentage: 8%) at an O_2 tension of 0 mmHg.

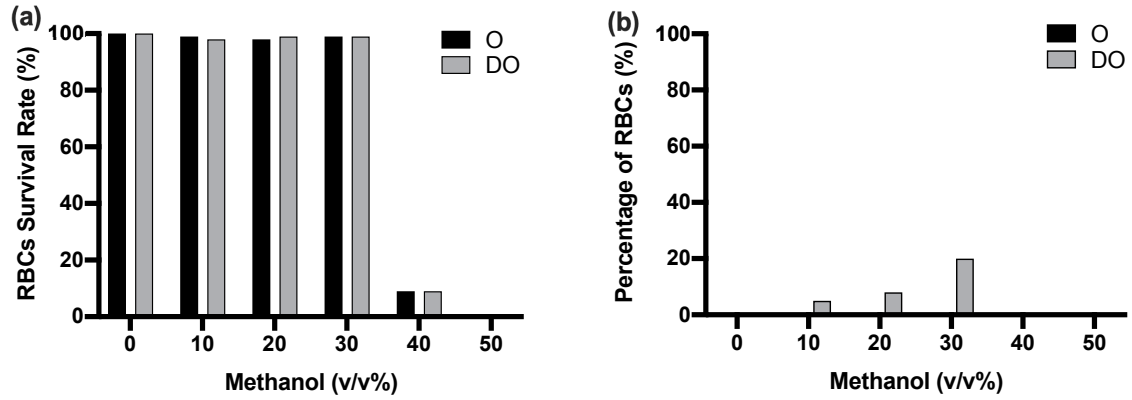

Figure 4.6.1 Effect of methanol on fixation of HbSS RBCs. HbSS RBCs (1% Hct) were first equilibrated in Eschweiler tonometers for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. Different volumes of chilled methanol were then added into aliquots which were then incubated for 10 min before cell counting under microscope. Methanol were placed on ice and bubbled with desired O_2 tensions before utilising. Figure (a) represents the survival rate of HbSS RBCs under different volume combination of chilled methanol / Cl-MBS. Figure (b) represents sickling percentage of RBCs founded in aliquots after 10 min incubation with desired methanol concentration. "O" represents HbSS RBCs were flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs were flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. Histograms represent a single experiment representative of 2 others.

To examine whether 30% methanol (v/v%) could adequately fix RBCs in all three media (Cl-MBS, BBS and plasma), methanol was added to RBC aliquots which had been fully oxygenated or deoxygenated for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. Figure 4.6.2 shows that there was no significant change in sickling percentage comparing RBCs incubated in the three buffers at either O_2 tension. The

result suggested that RBCs in Cl-MBS and BBS presented the same sickling percentage in 30% methanol as they do in 0.3% glutaraldehyde. Hence, 30% chilled methanol was used for cell fixation in subsequent experiments in this section.

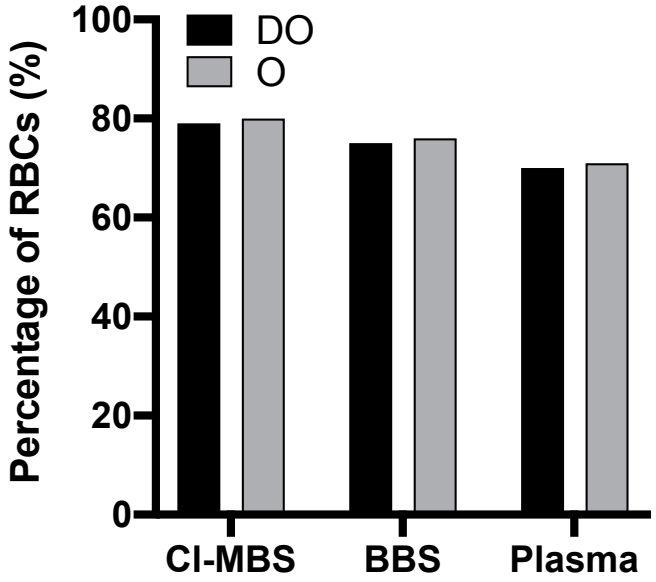

Figure 4.6.2 Effect of RBC fixation by 30% methanol (v/v%) in Cl-MBS, BBS and plasma. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. Methanol was placed on ice and bubbled with desired O_2 tensions. Chilled methanol (30 v/v%) was then added into RBC aliquots which were then incubated for a further 10 min before cell counting. Methanol aliquots were placed on ice and bubbled with desired O_2 tensions before utilising. Experimental pH was pH 7.4, adjusted using HNO_3 in Cl-MBS, but with PCO_2 = 43 mmHg in the case of BBS and plasma. After addition of methanol, RBC suspensions were then flushed with 150 mmHg or 0 mmHg O_2. "O" represents HbSS RBCs flushed with 150 mmHg air, in which RBCs were fully oxygenated, "DO" represents flushed with 150 mmHg N_2, in which RBCs were fully deoxygenated. Histograms represent a single experiment representative of 2 others.

After establishing an appropriate protocol for fixation of RBCs using methanol, the effect of medium composition of Cl-MBS, BBS and plasma on RBC sickling was then investigated. RBC aliquots were incubated at oxygen tensions of 150 mmHg and 0 mmHg at pH 7.4. In Figure 4.6.3, RBC sickling was 6% ($p < 0.05$) and 8% ($p < 0.05$) less when RBC aliquots were incubated with BBS and plasma, compared to that in Cl-MBS. There was no significant change between HbSS RBCs incubated with BBS and autologous plasma at oxygen tension of 0 mmHg.

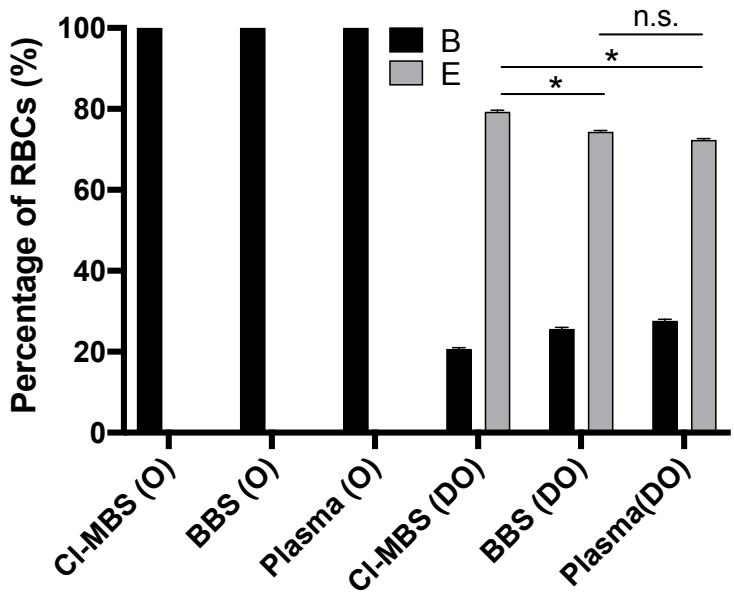

Figure 4.6.3 Effect of buffer (Cl-MBS, BBS and plasma) on sickling of HbSS RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in Cl-MBS, BBS and plasma for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. Experimental pH was pH 7.4, adjusted using HNO_3 in MBS, but with PCO_2 = 43 mmHg in the case of BBS and plasma. The aliquots were fixed by 30% chilled methanol as described in the legend to Figure 4.6.2. "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

4.3 Effect of lactic acid and low pH on sickling of HbSS RBCs

Acidosis is well known as a promoter of HbS polymerisation, however, only a few studies report acid / base disorders in SCA patients (Petto *et al.*, 2011). The commonest cause of physiological acidosis is anaerobic metabolism in skeletal muscle beds with glycolysis producing high levels of lactic acid. The membrane of RBCs is known to express monocarboxylate transporters which will enable lactic acid or lactate to gain access to their cytoplasm. Notwithstanding, the effect of acidosis induced by lactic acid has not been hitherto investigated on HbS polymerisation and RBC shape change. In this series of experiments, therefore, the sickling of HbSS RBCs was investigated in the absence / presence of lactic acid (10 mM) over a wide range of pHs (pH 7.4, pH 7.0, pH 6.5 and pH 6.0) in Cl-MBS. In Figure 4.7 (a), in the absence / presence of lactic acid, no sickling percentage was observed in HbSS RBCs at an oxygen tension of 150 mmHg.

At an oxygen tension of 0 mmHg (Figure 4.7 (b)), in the presence 10 mM lactic acid, sickling percentages of deoxygenated HbSS RBCs increased by 85% ($p < 0.05$) at pH

7.4 Cl-MBS, 60% ($p < 0.05$) at pH 7.0 Cl-MBS, 47% ($p < 0.05$) at pH 6.5 Cl-MBS and 32% ($p < 0.05$) at pH 6.0 Cl-MBS, compared to that in the absence of lactic acid, showing that lactic acid resulted in greater morphological change.

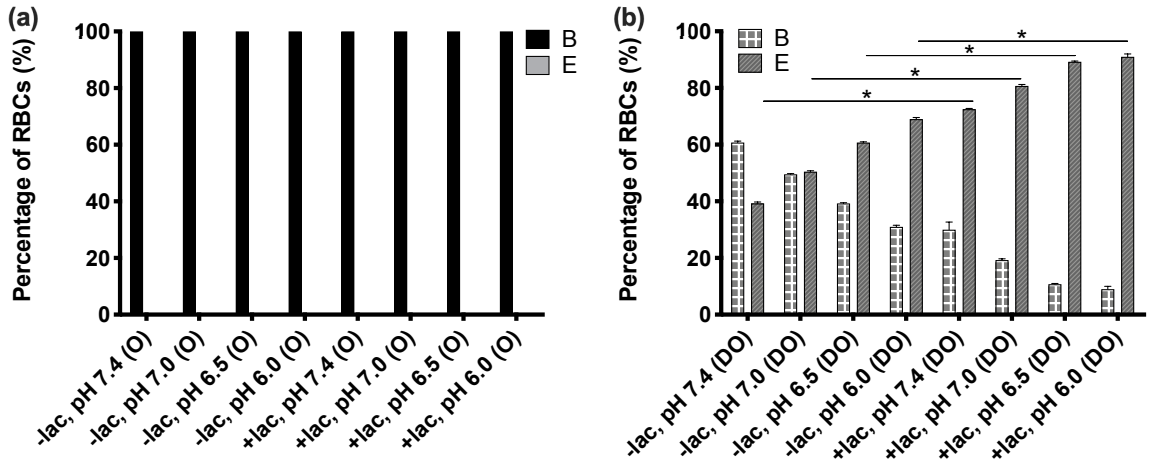

Figure 4.7 Effect of lactic acid and a range of extracellular pH on sickling of HbSS RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in Cl-MBS at different extracellular pHs in the absence / presence of 10 mM lactic acid (Figure 4.7 (a) and Figure 4.7 (b)) for 15 min at O_2 tensions of 150 mmHg and 0 mmHg, respectively. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "-lac" represents HbSS RBCs were incubated in Cl-MBS in the absence of lactic acid; "+lac" represents HbSS RBCs were incubated in Cl-MBS in the presence of 10 mM lactic acid; "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

4.4 Effect of incubation time and extracellular pH on sickling

Sickling of HbSS RBCs was investigated following incubation for different durations (0, 20 and 40 min) in pH 7.0 Cl-MBS at 37 °C.

There was no sickling apparent at 150 mmHg oxygen among duration of incubation (0, 20 or 40 min) (Figure 4.8). At 0 mmHg oxygen, at pH 7, sickling percentages were markedly increased after 20 min and 40 min, being 87% ($p < 0.05$) and 88% ($p < 0.05$) higher (Figure 4.8).

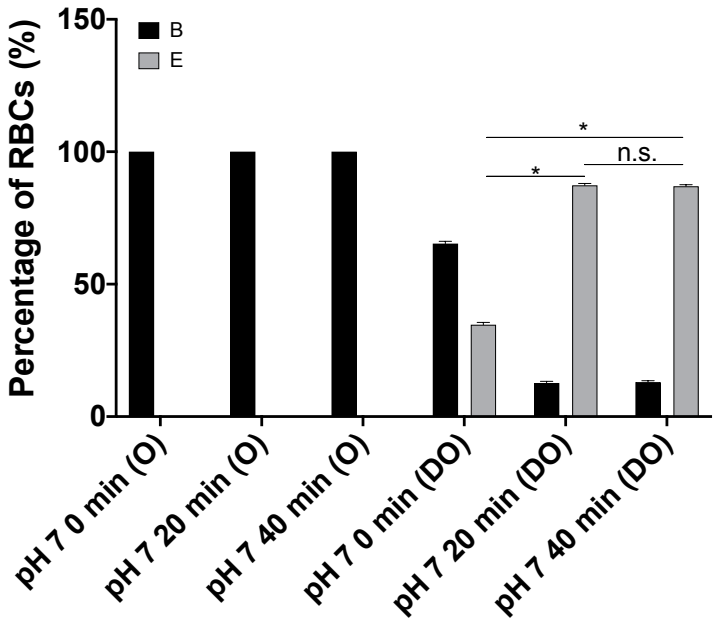

Figure 4.8 Effect of duration of incubation on sickling of HbSS RBCs at different extracellular pHs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in Cl-MBS (pH 7.0) for 0, 20 and 40 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed accordingly at desired time course as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs flushed with 150 mmHg O_2, in which RBCs were fully oxygenated; "DO" represents HbSS RBCs flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

4.5 Effect of modulation of protein phosphorylation by staurosporine, calyculin A, NEM and WNK463 on sickling

In subsequent work (chapter 7), the effect of pharmacological reagents which modulate protein phosphorylation were investigated to determine their effects on KCC activity (Chapters 6 and 7). It was therefore important to ascertain whether or not these compounds altered RBC morphology, which on its own may modify KCC activity.

4.5.1 Effect of staurosporine on sickling

The effect of staurosporine on sickling of HbSS RBCs was first investigated at pH 7.4 in Cl-MBS. There was no significant change in the presence of 100 μM staurosporine at pH 7.4 Cl-MBS, compared to that in its absence (Figure 4.9).

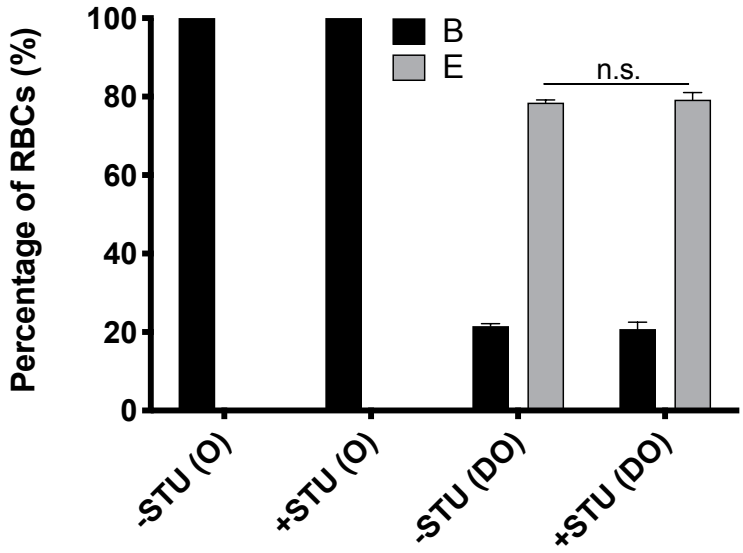

Figure 4.9 Effect of staurosporine on sickling of HbSS RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in the absence /presence of 100 μM staurosporine for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs were flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs were flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. n.s. indicates "not significant" by Student's t test (n=4).

4.5.2 Effect of calyculin A on sickling

The sickling of RBCs was investigated in the presence / absence of 100 nM calyculin A in pH 7.4 Cl-MBS at 37 °C. There was no significant change in the presence of 100 nM calyculin A at pH 7.4 Cl-MBS, compared to that in its absence (Figure 4.10).

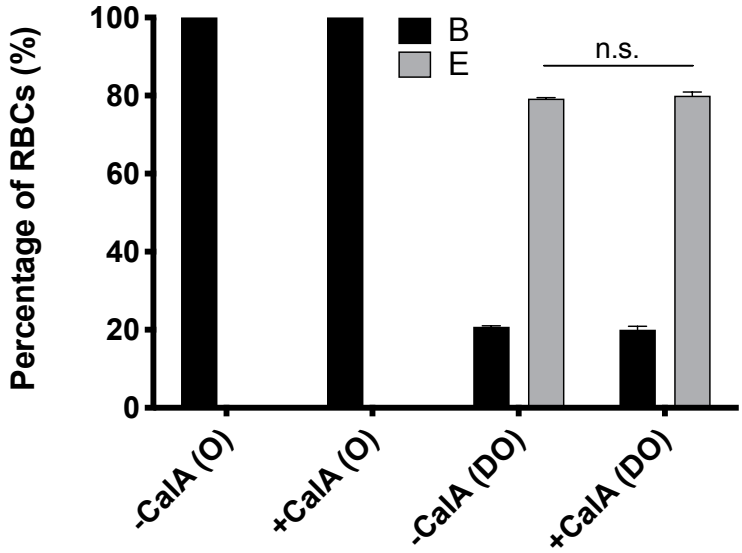

Figure 4.10 Effect of calyculin A on sickling of RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in the absence /presence of 100 nM calyculin A for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs were flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs were flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. n.s. indicates "not significant" by Student's t test (n=4).

4.5.3 Effect of NEM on sickling

The sickling of RBCs was investigated in the presence / absence of 1 mM NEM in pH 7.4 Cl-MBS at 37 °C. There was no significant change in the presence of 1 mM NEM at pH 7.4 Cl-MBS, compared to that in its absence (Figure 4.11).

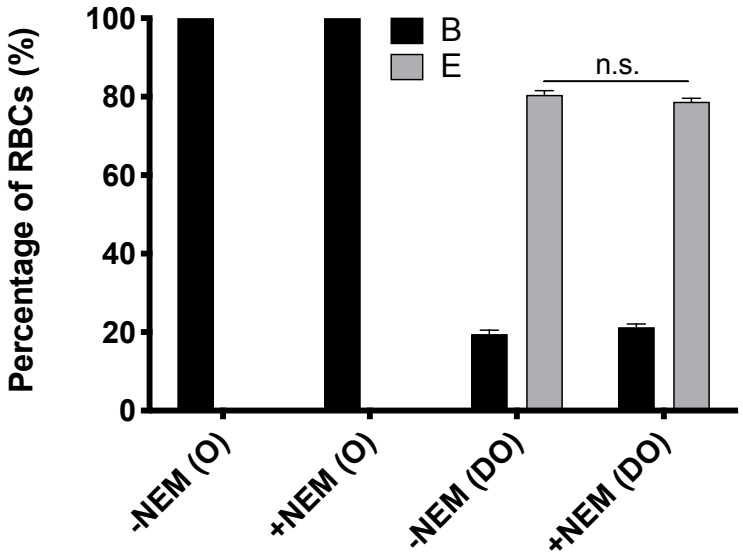

Figure 4.11 Effect of NEM on sickling of RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in the absence /presence of 1 mM NEM for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs were flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs were flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. n.s. indicates "not significant" by Student's t test (n=4).

4.5.4 Effect of WNK463 on sickling

The sickling of RBCs was investigated in the presence / absence of 40 nM WNK463 in pH 7.4 Cl-MBS at 37 °C. There was no significant change in the presence of 40 nM WNK463 at pH 7.4 Cl-MBS, compared to that in its absence (Figure 4.12).

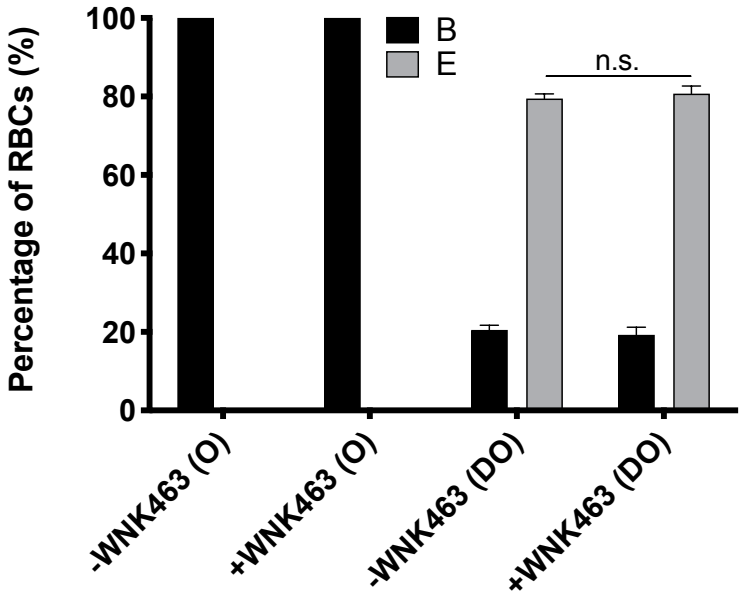

Figure 4.12 Effect of WNK463 on sickling of RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in the absence /presence of 40 nM WNK463 for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs were flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs were flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. n.s. indicates "not significant" by Student's t test (n=4).

4.6 Effect of cytochalasin B on sickling

The sickling of HbSS RBCs was investigated in the presence / absence of 10 μM cytochalasin B in pH 7.4 Cl-MBS at 37 °C. There was no significant change in the presence of 10 μM cytochalasin B at pH 7.4 Cl-MBS, compared to that in its absence (Figure 4.13).

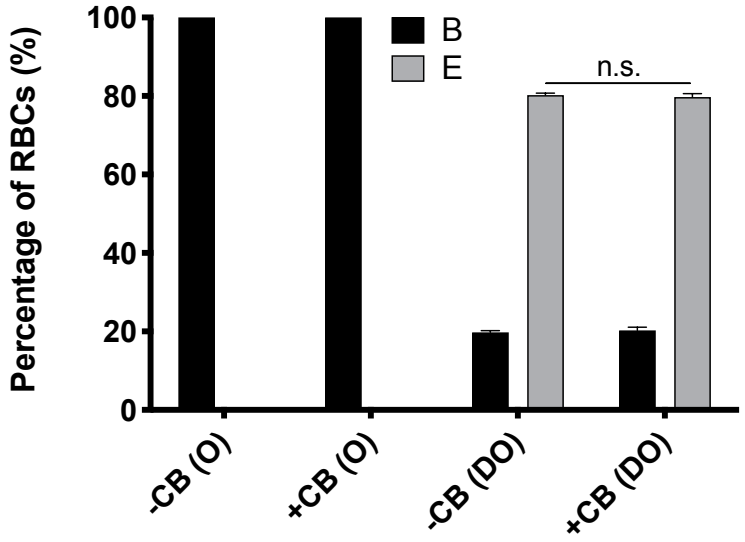

Figure 4.13 Effect of cytochalasin B on sickling of RBCs. HbSS RBCs (1% Hct) were firstly equilibrated in Eschweiler tonometers in the absence /presence of 10 μM cytochalasin B for 15 min at O_2 tensions of 150 mmHg and 0 mmHg. The aliquots were fixed as described in the legend to Figure 4.1. Herein, "O" represents HbSS RBCs were flushed with 150 mmHg O_2, in which RBCs were fully oxygenated, "DO" represents HbSS RBCs were flushed with 0 mmHg O_2, in which RBCs were fully deoxygenated. "B" represents biconcave HbSS RBCs and "E" represents elongated HbSS RBCs. Histograms represent means ± S.E.M. n.s. indicates "not significant" by Student's t test (n=4).

4.7 Discussion

4.7.1 Effect of physiological factors on sickling

The ability to deform repeatedly is an important physiological feature of RBCs as they pass through the microvasculature and splenic sinusoids, and it is generally agreed that lack of this ability leads to vascular occlusion and disruption of tissue oxygenation. In SCA patients, combination of RBC sickling and RBC adherence to the endothelium leads to various SCD complications such as acute chest syndrome, avascular necrosis and organ damage. However, there is more than one physiological factor causing polymerisation of HbS and inducing sickling of HbSS RBCs.

The rheologic properties of HbSS RBCs are influenced by various physiological factors such as oxygen tension, rate of deoxygenation, pH, urea, cell volume (surface to volume ratio, S/V ratio), buffer, lactic acid and low pH. This study re-emphasises the importance of these key physiological parameters which initiates sickling, together with possible methods for inhibition.

Previous studies of the rheologic properties of HbSS RBCs have focused on the function of oxygen tension (Nash *et al.*, 1986; Lu *et al.*, 2016) and rate of deoxygenation (Kaul & Xue, 1991; Rab *et al.*, 2019). The results summarised in Table 4.1 involving oxygen tension and rate of deoxygenation display trends similar to those reported in these previous studies (Roselli & Fatt, 1980; Du *et al.*, 2015; Rab *et al.*, 2019), notwithstanding the different conditions used, notably the use of Eschweiler tonometers. Sickling percentage of HbSS RBCs is deoxygenation-dependent, and increased greatly as oxygen tension was decreased. Results also suggest that sickling percentage of HbSS RBCs increases as rate of deoxygenation increases. Herein, the results in Table 4.1 support the relationship that sickling of aerated HbSS red cells in Cl-MBS depended upon Hb desaturation due to lowered oxygen tension and rate of deoxygenation - conditions which are commonly seen in athletes with SCD or at high altitude or in aeroplanes (Green *et al.*, 1971; Schmied & Borjesson, 2014). Findings are also probably relevant to individuals with sickle cell trait (HbAS) who are largely ignored in the literature.

The effect of altered extracellular pH on RBC sickling was also studied. The primary cause of sickling appears to be low pH and deoxygenation (Table 4.1). Results were consistent with the situations in a previous study (Lange *et al.*, 1951; Bookchin *et al.*,

1976; Ueda & Bookchin, 1984). Herein, possible counterparts of results in Table 4.1 are seen in alkalosis and metabolic acidosis, whose possible mechanisms are mediated via low pH which could induce polymerisation of HbS and also enhance P_{sickle} activity in deoxygenated HbSS RBCs (Joiner *et al.*, 1993) and hence encourage dehydration.

The effect of urea on sickling of HbSS RBCs, at concentrations found in the renal medulla during maximal antidiuresis, was also investigated. In Table 4.1, the result suggests that sickling percentage of HbSS RBCs decreases when urea concentration was increased under hypoxia. Possible mechanisms are that urea could have a weak-interaction with proteins by some other mechanisms (Makhatadze & Privalov, 1992), further stabilising soluble HbS and preventing sickling of HbSS RBCs.

The effect of hypotonicity on sickling of HbSS RBC was studied as well. In Table 4.1, it is summarised that sickling of HbSS RBC decreases when HbSS RBC volume increased. Possible mechanism is that increased cell volume will decrease intracellular [HbS] and further prevent red cell water loss and the efflux of K^+ (Bunn *et al.*, 1982; Fabry *et al.*, 1982; Eaton & Hofrichter, 1987).

The effect of incubation media, notably buffer composition, on sickling of HbSS RBCs were also studied in this chapter. In Table 4.1, the results suggest that BBS and plasma have a modest inhibitory effect on sickling of HbSS RBCs, compared to that in Cl-MBS. This might be due to that some of the composition of solutes in BBS and plasma - presumably the presence of bicarbonate or CO_2 in both – which may create an environment for preventing macromolecular crowding. The high activity of the RBC anion exchanger will enable rapid entry of bicarbonate to the cell interior.

The effect of lactic acid on sickling of HbSS RBCs was the final physiological parameter investigated. In a healthy human body, much of the lactate produced is buffered in the kidneys, heart muscle and mainly accumulated in the liver, active tissues and bloodstream. Especially in the heart muscle and liver tissue, two lactate molecules are transformed into glucose. In SCA patients, the baseline level of lactate is higher than normal values, leading to acute or chronic hepatic impairment and kidney dysfunction, which is thought to have a correlation between the accumulation of lactate in bloodstream and the hepatic lactate metabolism (Shao & Orringer, 1995; Banerjee *et al.*, 2001). In Table 4.1, sickling of HbSS RBCs was shown to increase after addition of 10 mM lactic acid, which suggests that sickling of HbSS RBCs not only correlates to

pH value, but also is dependent on the level of lactic acid. As for bicarbonate, the RBC monocarboxylate transporter will allow lactate to enter the cytoplasm where it may modulate HbS polymerisation in the opposite manner, promoting polymer formation. Overall these findings indicate that several physiological parameters may alter HbS polymerisation and be significant *in vivo* although they are rarely considered (Gladwin & Sachdev, 2012; Merlet *et al.*, 2019).

Table 4.1 Effect of physiological stimuli on sickling

Physiological stimuli	**Sickling**
PO_2	PO_2 ↓, sickling ↑
Rate of deoxygenation	Rate ↑, sickling ↑
pH	pH ↓, sickling ↑
Urea	Sickling ↓
Anisotonicity	Cell volume ↑, sickling ↓
Medium composition	Compared to Cl-MBS, sickling ↓ in BBS and plasma
Duration of incubation	When PO_2 ↓ and time ↑, then sickling ↑
Lactic acid	Sickling ↑

4.7.2 Effect of pharmacological reagents on sickling

Herein, five pharmacotherapeutic reagents (staurosporine, calyculin A, NEM, WNK463 and cytochalasin B) were used to investigate their effects on sickling of HbSS RBCs, as they are important tools for the study of KCC activity (see Chapter 7).

Staurosporine is normally used as a broad-spectrum protein kinase inhibitor and has been shown previously to stimulate KCC activity in HbSS RBCs. Calyculin A is an inhibitor on phosphatase activity, which reduces KCC activity (Almasry *et al.*, 2016). NEM is a pharmacological reagent which mainly stimulate K-Cl cotransporters (KCCs) in human erythrocytes, probably via inhibition of regulatory protein kinases (Bize *et al.*, 2000).

With regards to WNK463, the result summarised in Table 4.2 suggests that sickling of HbSS RBCs did not change after addition of WNK463. WNK463 is a pan-WNK inhibitor, which increase KCC activity by inhibiting WNK in WNK/SPAK/OSR1 pathway in HbSS RBCs (Lu *et al.*, 2019).

In Table 4.2, the summary of results shows that sickling of HbSS was unaffected by incubation with any of these reagents.

Overall, the action of these reagents on sickling imply that their effect on morphological shape of sickle cells need not be considered when examining their effects on KCC activity.

Finally, cytochalasin B is known to bind to actin filaments (Theodoropoulos *et al.*, 1994) and also inhibits the binding of G-actin to the cytoplasmic face of RBC membranes (Gokhin *et al.*, 2015). Also, it acts as an inhibitor of gas transporters such as hexose transport (Ebstensen & Plagemann, 1972) and some other actions relevant to RBC permeability and various signalling pathways including regulating protein kinase activity (Pedersen *et al.*, 2001). Though its site of action remains unclear, it did not alter sickling of HbSS RBCs (Table 4.2).

Table 4.2 Effect of pharmacological reagents on sickling

Pharmacological reagents	**Sickling**	**Actions of the drugs**
Staurosporine	n.s.	A protein kinase inhibitor, activate KCC in HbSS RBCs
Calyculin A	n.s.	A phosphatase inhibitor, activate KCC in HbSS RBCs
NEM	n.s.	A regulator protein kinase inhibitor, activate KCC in HbSS RBCs
WNK463	n.s.	A pan-WNK inhibitor, inhibit WNK andactivate KCC in HbSS RBCs
Cytochalasin B	n.s.	An inhibitor of the binding of G-actin to RBC membrane; an inhibitor of sugar transporters; altering some other protein kinases which regulates RBC permeability

n.s. represent "not significant".

5 Deoxygenation-induced phosphatidylserine exposure

5.0 Introduction

The externalisation of plasma membrane aminophospholipid phosphatidylserine (PS) in RBCs is a hallmark in those patients suffering from sickle cell anaemia, and occurs at a high level (~2-11%), compared to that in healthy individuals (<1% PS) (Wood *et al.*, 1996; De Jong *et al.*, 2001). The externalisation of PS in HbSS RBCs is believed to induce haemolysis and vaso-occlusion, and may be recognised by macrophages leading to the removal of RBC from the circulation. Although the mechanism of inducing PS exposure in HbSS RBCs remains unclear, deoxygenation-induced sickling (Lubin *et al.*, 1981) and an increased concentration of intracellular Ca^{2+} (Bitbol *et al.*, 1989; Weiss *et al.*, 2011) have been suggested to play an important role in SCA pathogenesis.

According to Lubin *et al.*, the level of PS in HbSS RBCs can be further increased / decreased by deoxygenation / reoxygenation *in vitro* (Lubin *et al.*, 1981), which suggests red cell shape change by HbS polymerisation and sickling might be involved. Also, the involvement of Ca^{2+} was postulated, and was investigated from the 1980s onwards. However, less attention has been directed towards investigating the role of other physiological stimuli and potential pharmacological modulators. These factors represent the focus of the present chapter.

Investigation of the PS exposure mechanism is complicated work by the numerous stimuli involved, including various cell signalling pathways leading to apoptosis (Boas *et al.*, 1998), HbS instability and oxidative challenge (Obrador *et al.*, 2015) and inactivation of the ATP-dependent flippase or PMCA by decreased ATP levels or those of intracellular Mg^{2+} (Bitbol *et al.*, 1987; Etzion *et al.*, 1993). Moreover, the rate of deoxygenation alters the shape of HbSS RBCs; slow HbS polymerisation results in elongated RBCs, medium in holly leaf-like shapes and fast polymerisation results in granular RBCs (Mozzarelli *et al.*, 1987) and might alter the extent of activation of P_{sickle} and hence of the entry of Ca^{2+} (Lew *et al.*, 1997). Moreover, PS exposure can also be increased by HbSS RBC shrinkage following activation of the Gardos channel by elevated intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Lang *et al.*, 2003*a*).

In this chapter, some of the relevant physiological stimuli which affect PS exposure in HbSS RBCs, such as the deoxygenation rate, pH, urea (in combination with hypertonic NaCl) and incubation media / buffers, were examined. Moreover, pharmacological manoeuvres such as Ca^{2+} removal and reagents such as GsMTx-4 and ruthenium red were also investigated to determine their effects on PS exposure in HbSS RBCs.

5.1 Results

5.2 Loading of Ca^{2+} on PS scrambling in HbSS RBCs

In this experiment, the effect of different $[Ca^{2+}]_o$s (0, 0.1, 0.3, 0.6, 1 and 10 μM) on the percentage of RBCs exposing PS in HK saline was carried out in RBCs treated with the ionophore Br-A23187. PS scrambling rose markedly with the increase in $[Ca^{2+}]_o$s from 0 to 10 μM Ca^{2+} (Figure 5.1), in agreement with previous work carried out in this laboratory (Weiss *et al.*, 2011; Cytlak *et al.*, 2013). In Figure 5.1, compared to the percentage PS exposure in low micromolar $[Ca^{2+}]_o$s (0 - 1 μM), the PS scrambling percentage was 10-fold greater in high micromolar $[Ca^{2+}]_o$s (10 μM).

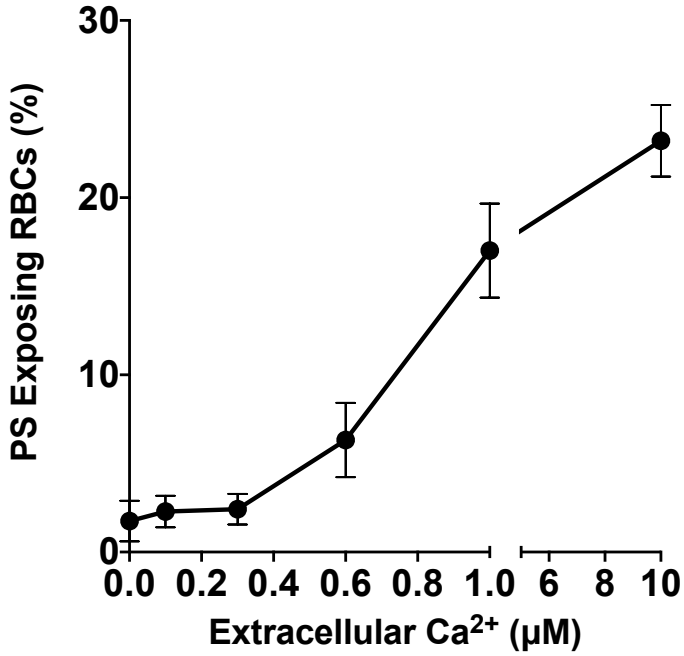

Figure 5.1 Loading of Ca^{2+} on PS scrambling in HbSS RBCs. RBCs (0.5% Hct) were first treated with vanadate (1 mM) to inhibit the flippase and also the plasma membrane Ca^{2+} pump. Incubation was carried out in HK saline with the addition of Br-A23187 (6 μM) and EGTA/Ca^{2+} mixtures for 3 min at 37 °C. RBCs aliquots were added to lactadherin – labelling solution (HK saline containing 16 nM LA-FITC). Labelling of PS exposing RBCs was carried out for 10 min in the dark at room temperature after which RBCs were rinsed once (600 *x g* for 3 min, followed by a short spin at 16,000 g for 10 s), labelled with LA-FITC and kept on ice until FACS analysis. Symbols represent means ± S.E.M (n =3).

5.3 Effect of deoxygenation rate on PS scrambling in HbSS RBCs

In Figure 5.2, the effect of deoxygenation rate (adjusted by controlling rotation speed of Eschweiler tonometer) on PS scrambling in HbSS RBCs was investigated. RBCs were incubated in Cl-MBS at pH 7.4. No significant PS exposure was present at the start of the experiment (time 0 min). Percentage PS exposure at a tonometer rotation speed of 180 rpm was markedly higher than at rotation speeds of 100 rpm and 80 rpm by 46% ($p < 0.05$) and 1130%, respectively, after a 40 min incubation.

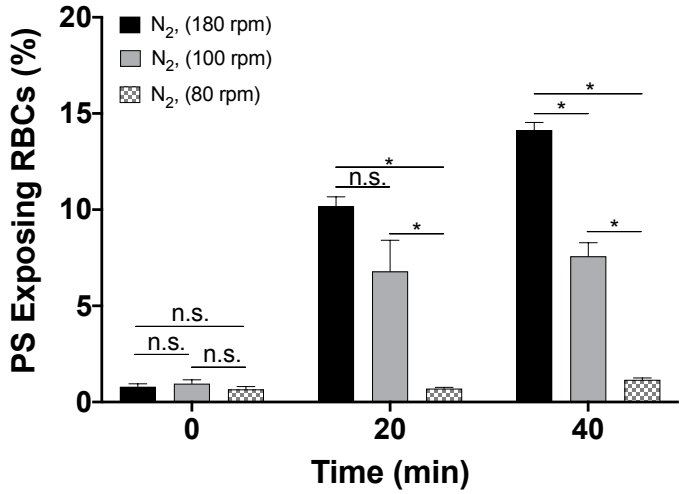

Figure 5.2 Effect of deoxygenation rate on PS scrambling in HbSS RBCs. RBCs (0.5% Hct) were first treated with vanadate (1 mM) to inhibit the plasma membrane Ca^{2+} pump and the flippase. Incubation was carried out in Cl-MBS for 0, 20 and 40 min at 37 °C with different rotation speeds - 180 rpm, 100 rpm and 80 rpm – at 0 mmHg O_2, supplemented with N_2. Lactadherin-labelling of PS exposing RBCs was carried out for 10 min in the dark at room temperature after which RBCs were washed (600 *x g* for 3 min, followed by a short spin at 16,000 *x g* for 10 s), labelled with LA-FITC and kept on ice until FACS analysis. Symbols represent means ± S.E.M (n =3).

5.4 Effect of pH on PS scrambling in HbSS RBCs

In this experiment, the effect of pH on PS scrambling in HbSS RBCs was investigated. In Figure 5.3, no significant percentage PS exposure in HbSS RBCs was found at either pH 8.0, pH 7.4 or pH 6.0 Cl-MBS at the start of incubation in Cl-MBS (0 min). At pH 7.4, percentage of PS exposure increased with time after 60 min incubation, which increased by 39% compared to that at the start of incubation (0 min).

Moreover, percentage PS exposure of HbSS RBCs was 60% ($p < 0.05$) higher in pH 7.4 Cl-MBS at oxygen tension of 0 mmHg compared to that in pH 8.0 Cl-MBS after 60 min incubation. Percentage PS exposure of HbSS RBCs incubated with pH 7.4 Cl-MBS was 58% ($p < 0.05$) lower than that in pH 6.0 Cl-MBS after 60 min incubation.

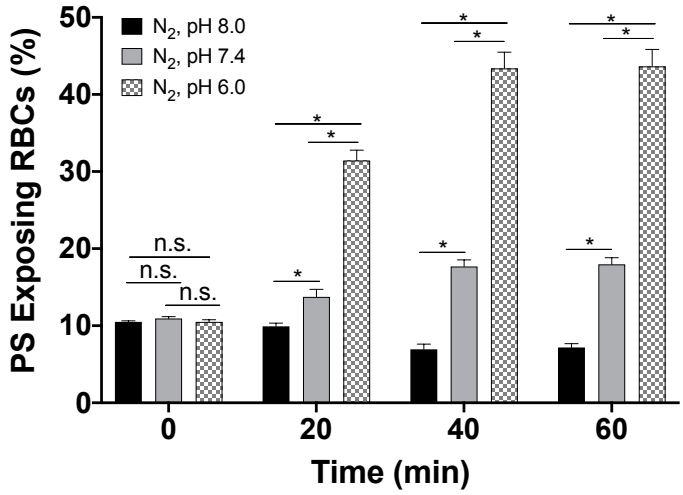

Figure 5.3 Effect of pH on PS scrambling in deoxygenated HbSS RBCs. RBCs (0.5% Hct) were incubated at 37 °C for 60 min at pH 8.0, pH 7.4 or pH 6.0 in Cl-MBS, which contained with 1 mM vanadate and 1.1 mM Ca^{2+}. The same FACS analysis procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

5.5 Effect of urea on PS scrambling in HbSS RBCs

Effect of urea on PS scrambling in HbSS RBCs incubated at pH 7.4 in Cl-MBS was investigated. In Figure 5.4, PS exposure at the start of the incubation was low, < 5%. In the absence of urea, PS exposure increased progressively with duration of deoxygenation reaching a value of about 40 % after 80 min. However, no significant percentage PS exposure was observed among HbSS RBCs incubated with added urea at any of the concentrations tested (200, 600 or 1200 mM).

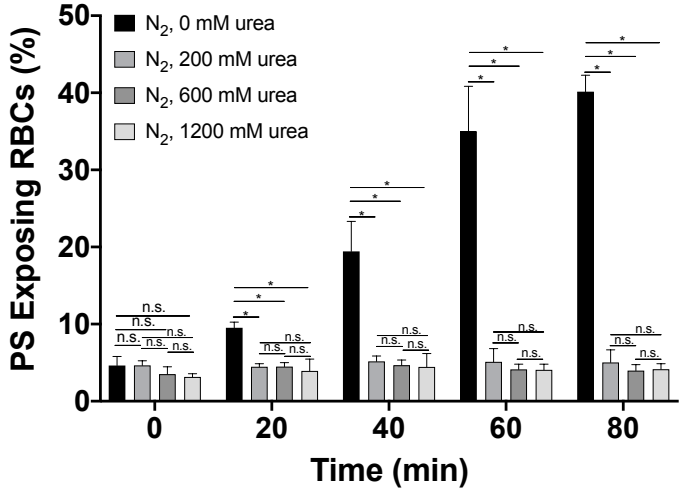

Figure 5.4 Effect of urea on PS scrambling in deoxygenated HbSS RBCs. RBCs (0.5% Hct) were incubated at 37 °C for 60 min in pH 7.4 Cl-MBS, which contained with 1 mM vanadate and 1.1 mM Ca^{2+}. The same FACS analysis procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

5.6 Effect of urea, hypertonicity and extracellular pH on PS scrambling in HbSS RBCs

Effect of urea on PS scrambling in deoxygenated HbSS RBCs incubated at different extracellular pHs was investigated. In Figure 5.5, percentage PS exposure in HbSS RBCs incubated at pH 8.0 in Cl-MBS containing 600 mM urea and 300 mM NaCl was 89% ($p < 0.05$) lower than that in the absence of urea and isotonic (145 mM) NaCl at 0 min. There was no significant change of percentage PS exposure in HbSS RBCs incubated with pH 8.0 Cl-MBS in the presence / absence of 600 mM urea and 300 mM NaCl over an 80 min time course.

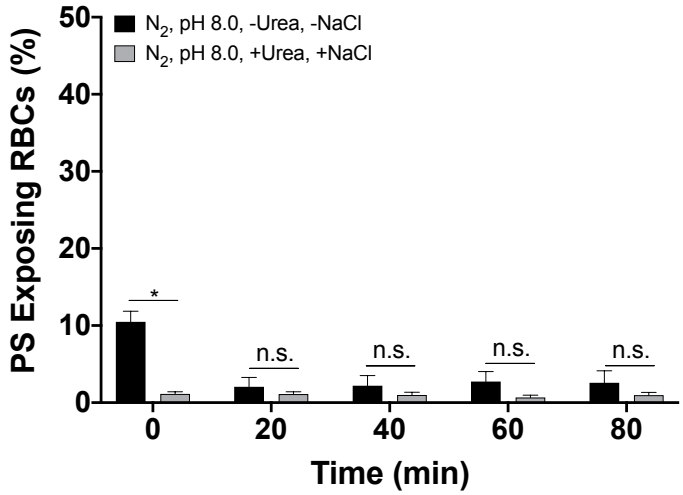

Figure 5.5 Effect of urea on PS scrambling in deoxygenated HbSS RBCs incubated at pH 8.0 in Cl-MBS. RBCs (0.5% Hct) were incubated at 37 °C for 80 min in pH 8.0, Cl-MBS, in the presence / absence of 600 mM urea and 300 mM NaCl. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

In Figure 5.6, effect of urea on PS scrambling in HbSS RBCs incubated at pH 7.4 in Cl-MBS, in the presence / absence of urea and NaCl, was investigated. Percentage PS exposure in HbSS RBCs at pH 7.4 in Cl-MBS containing 600 mM urea and 300 mM NaCl was 76% ($p < 0.05$) lower than that in the absence of urea and NaCl at 0 min. Also, percentage PS exposure in HbSS RBCs, incubated in pH 7.4 Cl-MBS containing 600 mM urea and 300 mM NaCl, was 88% ($p < 0.05$) lower than that in the absence of urea and in isotonic NaCl after 80 min incubation.

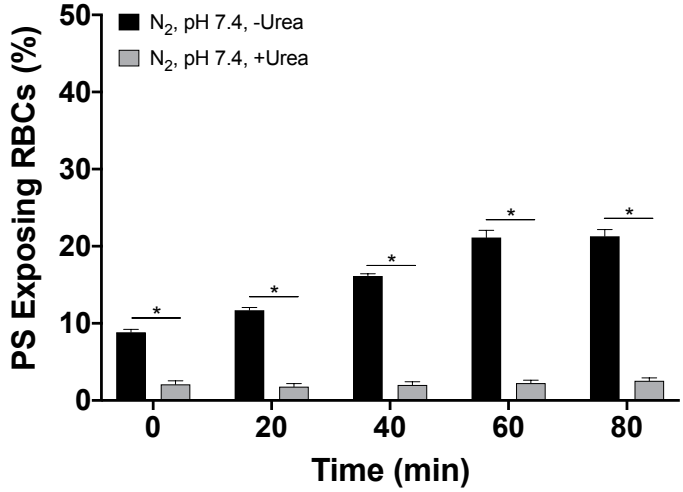

Figure 5.6 Effect of urea on PS scrambling in deoxygenated HbSS RBCs incubated at pH 7.4 in Cl-MBS. RBCs (0.5% Hct) were incubated at 37 °C for 80 min in pH 7.4, Cl-MBS, which was in the presence / absence of 600 mM urea and 300 mM NaCl. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

In Figure 5.7, effect of urea on PS scrambling in HbSS RBCs incubated in pH 7.0 Cl-MBS, in the presence / absence of urea and NaCl, was investigated. Percentage PS exposure in HbSS RBCs, incubated at pH 7.0 in Cl-MBS containing 600 mM urea and 300 mM NaCl, was 84% ($p < 0.05$) lower than that in the absence of urea and NaCl at 0 min. Also, percentage PS exposure in HbSS RBCs, incubated at pH 7.0 in Cl-MBS containing 600 mM urea and 300 mM NaCl, was 95% ($p < 0.05$) lower than that in the absence of urea and NaCl after 80 min incubation.

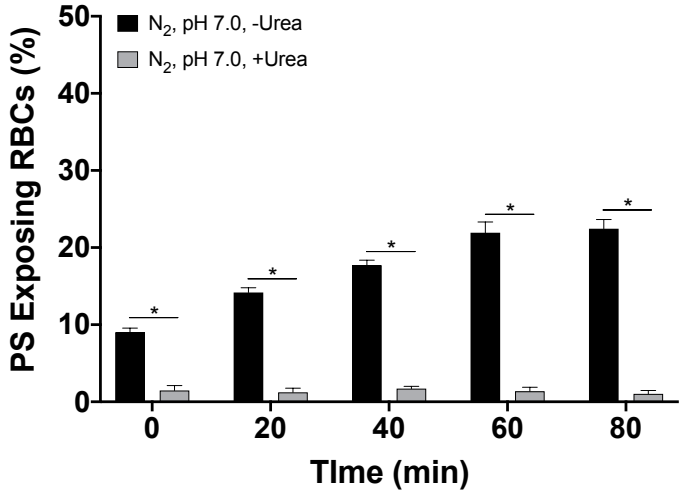

Figure 5.7 Effect of urea on PS scrambling in deoxygenated HbSS RBCs incubated at pH 7.0 in Cl-MBS. RBCs (0.5% Hct) were incubated at 37 °C for 80 min at pH 7.0 in Cl-MBS, which was in the presence / absence of 600 mM urea and 300 mM NaCl. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

In Figure 5.8, effect of urea on PS scrambling in HbSS RBCs incubated at pH 6.0 in Cl-MBS, in the presence / absence of urea and NaCl, was investigated. Percentage PS exposure in HbSS RBCs, incubated at pH 6.0 in Cl-MBS containing 600 mM urea and 300 mM NaCl, was 93% ($p < 0.05$) lower than that in the absence of urea and NaCl at 0 min. Also, percentage PS exposure in HbSS RBCs, incubated at pH 6.0 in Cl-MBS containing 600 mM urea and 300 mM NaCl, was 96% ($p < 0.05$) lower than that in the absence of urea and NaCl after 80 min incubation.

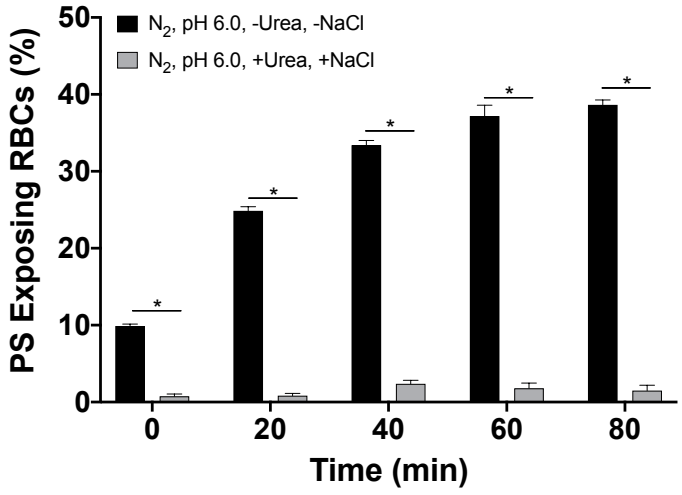

Figure 5.8 Effect of urea on PS scrambling in deoxygenated HbSS RBCs incubated at pH 6.0 in Cl-MBS. RBCs (0.5% Hct) were incubated at 37 °C for 80 min at pH 6.0 in Cl-MBS, which was in the presence / absence of 600 mM urea and 300 mM NaCl. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

In Figure 5.9, effect of urea on PS scrambling in HbSS RBCs incubate at pH 8.0, pH 7.4, pH 7.0 and pH 6.0, in the absence of urea and NaCl, were compared. There was no significant difference of percentage of PS exposure in HbSS RBCs incubated with pH 8.0, pH 7.4, pH 7.0 and pH 6.0 Cl-MBS in the absence of 600 mM urea and 300 mM NaCl at 0 min.

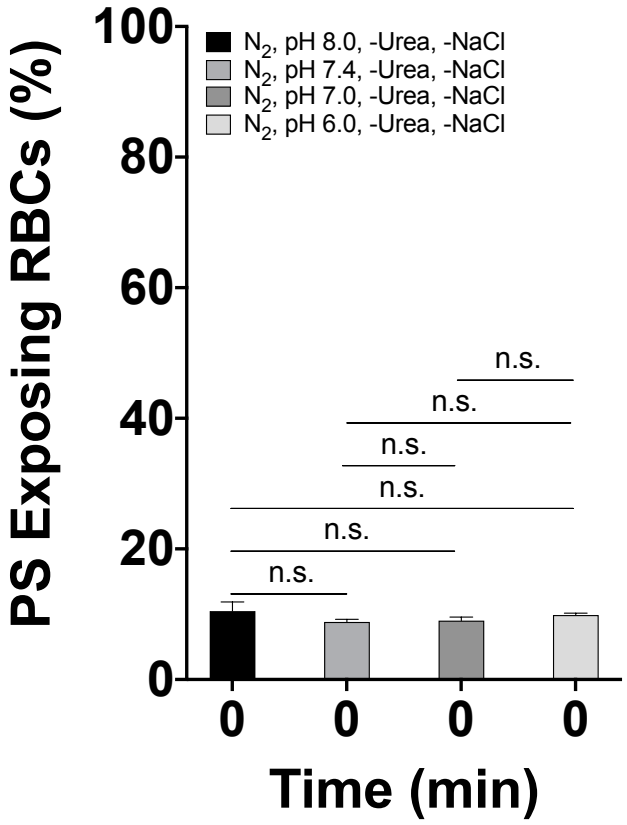

Figure 5.9 Effect of pH on PS scrambling in deoxygenated HbSS RBCs incubated at pH 8.0, pH 7.4, pH 7.0 and pH 6.0 Cl-MBS. RBCs (0.5% Hct) were incubated at 37 °C for 80 min in pH 8.0, pH 7.4, pH 7.0 and pH 6.0, Cl-MBS, in the absence of 600 mM urea and 300 mM NaCl. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3), n.s. indicates "not significant" by Student's t test.

5.7 Effect of incubation media on PS scrambling in HbSS RBCs

In Figure 5.10, the effect of buffer on PS scrambling in deoxygenated HbSS RBCs was investigated. PS exposure at time zero was 8 % and was the same in all three buffers. PS exposure then increased progressively in Cl-MBS reaching 33 % after 80 min. At all time points (20 - 80 min), PS exposure was reduced in BBS and plasma compared to that in Cl-MBS, with reductions of 43%, 47%, 52% and 51% (BBS), reductions of 43%, 50%, 47% and 47% (plasma), after 20, 40, 60 and 80 min incubation, respectively. There was no significant difference in PS exposure between RBCs incubated in BBS or plasma.

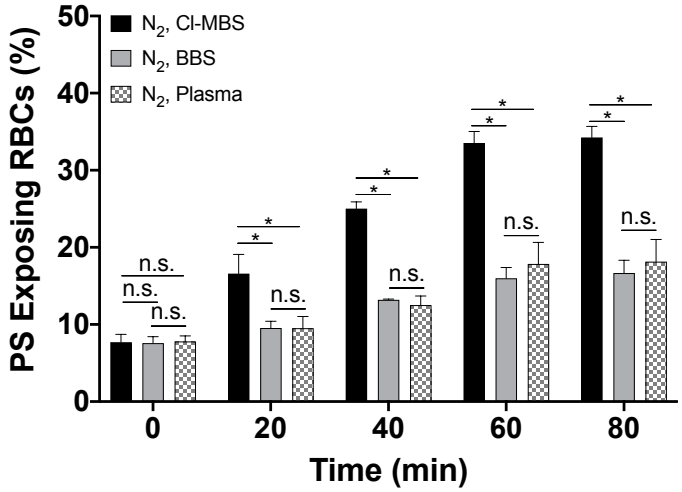

Figure 5.10 Effect of buffer on PS scrambling in deoxygenated HbSS RBCs. The same procedure as described in Figure 5.2 was followed but HbSS RBCs were incubated in three different incubation media: Cl-MBS, BBS and plasma. Herein, pH value of Cl-MBS was adjusted by utilising HNO_3. However, to achieve pH 7.4 in BBS/plasma, these media were gassed with the appropriate partial pressure of CO_2 (PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg) at 37 °C. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

5.8 Effect of extracellular [K^+] on PS scrambling in HbSS RBCs

In this experiment, the effect of extracellular [K^+] on PS scrambling in deoxygenated HbSS RBCs was investigated. Again, PS exposure increased progressively with time from 12 % at time zero reaching about 38 % after 80 min. There was no significant change in percentage PS exposure when HbSS RBCs were incubated at high extracellular [K^+] (100 mM [K^+]) Cl-MBS compared to that at low extracellular [K^+] (7.5 mM [K^+]) at any time points (up to 80 min).

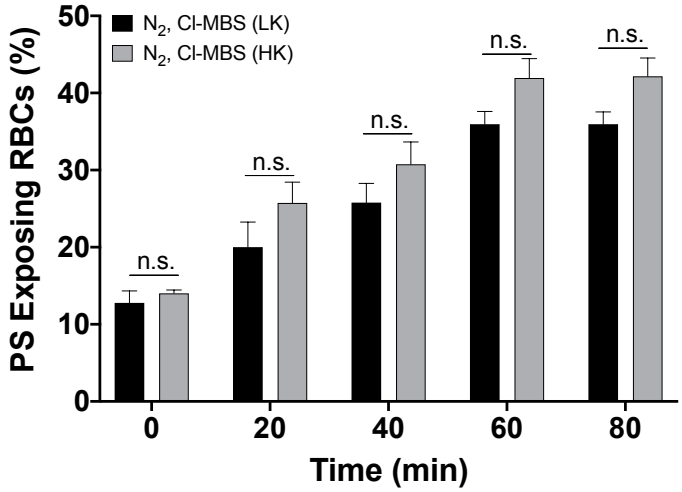

Figure 5.11 Effect of extracellular [K^+] on PS scrambling in deoxygenated HbSS RBCs. RBCs (0.5% Hct) were incubated at 37 °C for 80 min at pH 7.4 in Cl-MBS, which contained 7.5 mM [K^+] (LK, low extracellular [K^+] in Cl-MBS) or 100 mM [K^+] (HK, high extracellular [K^+] in Cl-MBS). The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. n.s. indicates "not significant" by Student's t test (n=3).

5.9 Effect of extracellular Ca^{2+} on PS scrambling in HbSS RBCs

The effect of extracellular Ca^{2+} on PS scrambling in deoxygenated HbSS RBCs was investigated. In Figure 5.12, there was no significant difference in percentage PS exposure in HbSS RBCs incubated with Cl-MBS in the absence / presence of Ca^{2+}, using either Cl-MBS containing 1.1 mM Ca^{2+} or Ca^{2+}-free Cl-MBS containing 1 mM EDTA) at 0 min, with levels of 12% in both conditions. With time, PS exposure increased to 42% in the presence of Ca^{2+} but only 22% in its absence. When Ca^{2+} was absent, PS exposure was reduced by 35%, 34%, 29% and 27% after 20, 40, 60 and 80 min, respectively.

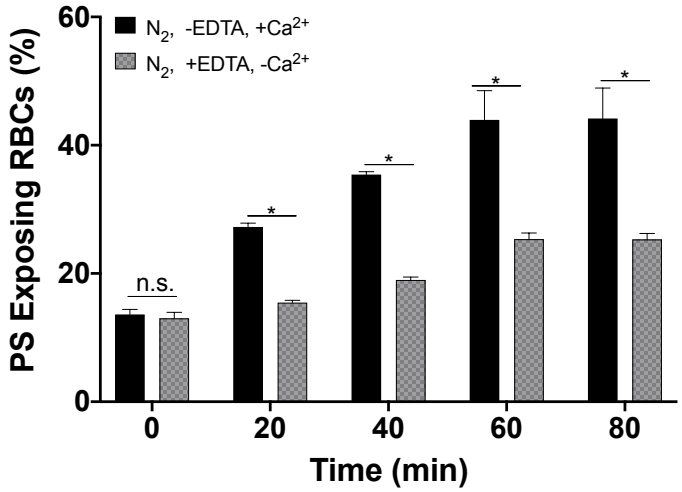

Figure 5.12 Effect of extracellular Ca^{2+} on PS scrambling in deoxygenated HbSS RBCs. RBCs (0.5% Hct) were incubated at 37 °C for 80 min at pH 7.4 in Cl-MBS, in the presence of Ca^{2+} (1.1 mM) or its absence (0 Ca^{2+} plus 1 mM EDTA). The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

5.10 Effect of GsMTx-4 on PS scrambling in HbSS RBCs

In this experiment, GsMTx-4, an inhibitor of Piezo1, was utilised to examine its effect on PS scrambling in HbSS RBCs, as Piezo1 is a candidate protein for the P_{sickle} conductance pathway activated by sickling. In Figure 5.13, deoxygenation again caused a time-dependent increase in PS exposure, reaching 17% after 80 min. At all time points, increased PS exposure was observed in deoxygenated Cl-MBS containing 10 μM GsMTx-4. GsMTx4 (10 μM) significantly increased percentage PS exposure by 106% after 80 min incubation. This finding was contrary to that expected.

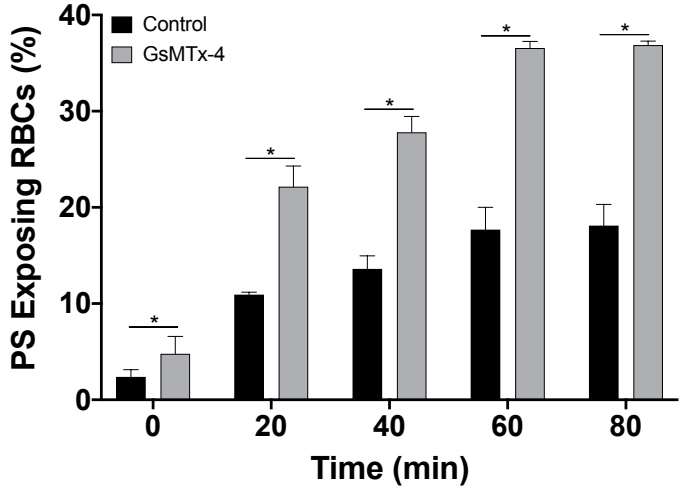

Figure 5.13 Effect of GsMTx-4 on PS scrambling in deoxygenated HbSS RBCs. RBCs (0.5% Hct) were incubated at 37 °C for 80 min at pH 7.4 in Cl-MBS, in the absence / presence of 10 μM GsMTx-4. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

5.11 Effect of ruthenium red on PS scrambling in HbSS RBCs

Because of the unexpected stimulatory effect of GsMTx-4 on PS exposure, in this experiment, the effect of ruthenium red was investigated. Ruthenium red is known to block both Piezo1 and Piezo2 (Coste *et al.*, 2010) as well as a number of TRPV channels which have also been postulated to be present in the RBC membrane – although it is a somewhat promiscuous reagent with other non-specific effects. Findings were similar to those observed with GsMTx-4. Increased PS exposure was observed in RBCs deoxygenated in Cl-MBS containing 30 μM ruthenium red. In the presence of ruthenium red, PS exposure was increased from 18% to 32% in deoxygenated RBCs after 80 min incubation.

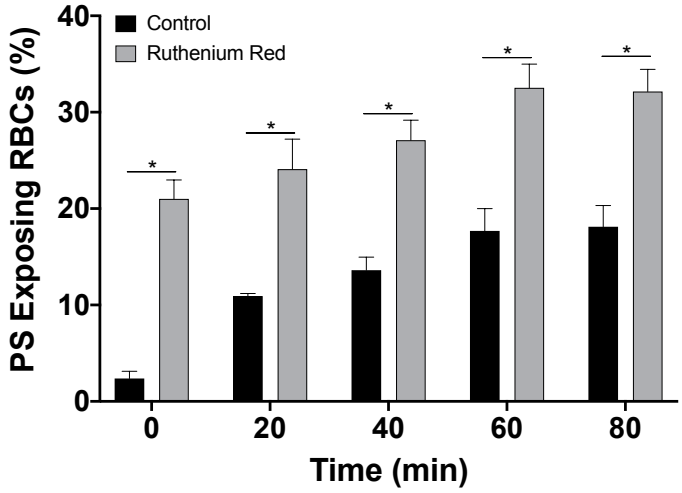

Figure 5.14 Effect of ruthenium red on PS scrambling in deoxygenated HbSS RBCs. RBCs (0.5% Hct) were incubated at 37 °C for 80 min at pH 7.4 in Cl-MBS, in the absence / presence of 30 μM ruthenium red. The same procedure as described in Figure 5.2 was then followed. Histograms represent means ± S.E.M. * $p < 0.05$ (n=3).

5.12 Discussion

The present findings further define the effects of various physiological stimuli and pharmacological agents on PS exposure in HbSS RBCs. PS exposure was found in various physiological conditions to which RBCs from SCA patients are likely to be exposed (Wood *et al.*, 1996; De Jong *et al.*, 2001; Dasgupta & Thiagarajan, 2005; Cytlak *et al.*, 2013) and which may therefore play a role in pathogenesis. Particularly, effects of some key physiological stimuli were examined such as deoxygenation rate, pH, urea (with high [NaCl]) and incubation media. Also, three typical pharmacological reagents, EDTA, GsMTx-4 and ruthenium red, were examined for their effects on PS exposure in HbSS RBCs.

5.12.1 Effect of physiological stimuli on PS scrambling in HbSS RBCs

Table 5.1 shows effects of some key physiological stimuli on PS scrambling in HbSS RBCs. In this study, PS scrambling in HbSS RBCs increased when the deoxygenation rate increased. Deoxygenation alone is well recognised to elicit PS exposure (Lubin *et al.*, 1981; Blumenfeld *et al.*, 1991). The current findings show rate of deoxygenation is also important. The exact mechanism of this requires further investigation, however, faster deoxygenation rate is known to cause increased morphological changes in sickle cells (chapter 4). It is possible that a more marked distortion of the membrane will cause greater activation of P_{sickle} and hence Ca^{2+} entry. Other possibilities include increased ceramide production which is also known to follow RBC distortion, notably shrinkage, and which is implicated in PS exposure (Lang *et al.*, 2003*b*). It was noticeable that in all experiments sickling was already maximal after 20 min deoxygenation, whilst significant PS exposure required at least 20 – 40 min. The reason of causing this delay might be that deoxygenation-induced process of accumulating sufficient $[Ca^{2+}]_i$ to reach the threshold for PS scrambling may be relatively slow, particularly in comparison with Ca^{2+} loading using an ionophore such as A23187 (Bitbol *et al.*, 1987; Williamson *et al.*, 1992; Kamp *et al.*, 2001).

Table 5.1 also shows the effect of pH on PS exposure in HbSS RBCs. The result indicates that PS scrambling increases as pH decreases over the range of pH 8.0 to pH 6.0. It is likely pH will fall to these low values in active muscle beds and in the renal medulla, hence contributing to vaso-occlusive crises which are associated with

exercise and with sickle nephropathy in which necrosis of the renal medulla is a common complication of the disease. Interestingly, although hypertonicity due to high urea levels - whether alone or in combination with high salt levels - reduced PS levels. This suggests that in a fully functioning kidney, urea may ameliorate the deleterious effects of the peculiar conditions found in the medulla during antidiuresis, at least in sickle patients. It is intriguing to speculate that the same dehydration protection effect will be found in RBCs from normal individuals. High urea levels, therefore, may have a dual role: their known effect to boost the countercurrent multiplier to maximise medullary hypertonicity for water conservation, but a second role in protecting RBCs as they traverse the vasa recta. In sickle nephropathy, as the renal medulla necroses and fibroses and becomes non-functional, this second protective effect of urea will be lost.

Finally, in the context of the relatively slow time course for PS exposure noted above, the low flow rates, extreme conditions and increased stickiness of sickle RBCs all potentially act synergistically to result in medullary infarction.

The effect of incubation media on PS exposure in HbSS RBCs is also summarised in Table 5.1. PS scrambling in HbSS RBCs was reduced in BBS and plasma, in comparison to that in Cl-MBS. In this context, it has been reported that a component of plasma, likely bicarbonate or CO_2, reduces KCC activity in RBCs relative to that observed in artificial saline (Godart *et al.*, 1997). The mechanism and significance of the present findings extend our appreciation of how RBCs behave *in vivo* and warrant further investigation.

Lastly, the effect of extracellular $[K^+]$ on PS exposure in HbSS RBCs was examined. There was no difference in PS exposure in HbSS RBCs in HK Cl-MBS or LK Cl-MBS although previously some inhibition has been observed in HK media (Weiss *et al.*, 2011). It remains unclear the mechanism that these differences with the present findings exist.

Table 5.1 Effect of physiological stimuli on PS scrambling in SCA patients.

Physiological stimuli	Phosphatidylserine exposure
Deoxygenation rate	Rate ↑, PS ↑
pH	pH ↓, PS ↑
Urea	PS ↓
Urea, in combination with NaCl	PS ↓
Incubation media	Compared to Cl-MBS, PS ↓ in BBS and plasma
Extracellular [K^+]	NC*

*Not changed.

5.12.2 Effect of pharmacological reagents on PS scrambling in HbSS RBCs

Table 5.2 summarises the effects of three pharmacological reagents on PS scrambling in HbSS RBCs.

EDTA is normally utilised as an anticoagulant reagent in blood samples meant for routine laboratory analysis (Kafka & Yermiahu, 1998). The rationale of anticoagulant is based on inhibiting the aggregation of platelet and various reaction of the haemostatic cascade by chelating free Ca^{2+} ions. In addition to its anticoagulant effect, the present result (Table 5.2) also show that EDTA has a marked inhibitory effect on PS exposure in HbSS RBCs, compared to those incubated in an extracellular [Ca^{2+}] of 1.1 mM. This has been observed previously (Weiss *et al.*, 2012; Cytlak *et al.*, 2013) and an obvious mechanism is the absence of entry of Ca^{2+} through the P_{sickle} pathway whose identity may be Piezo1 or possibly a TRPV channel. Accordingly, experiments were carried out with GsMTx-4, a spider venom neuropeptide which can inhibit cationic mechanosensitive channels (MSCs) including Piezo1 (Suchyna, 2017) and with ruthenium red which inhibits certain TRPV channels. Unexpectedly, results summarised in Table 5.2 show that both reagents actually increased PS exposure. It is difficult to reconcile these findings. However, GsMTx-4 is a difficult reagent and effects varies with batch. It also functions to distort cell membranes so whilst it may act as a Piezo1 inhibitor (Bae *et al.*, 2011), this action may be masked by membrane distortion rather increasing activity. Ruthenium red is also non-specific and it has been observed previously that it causes RBC clumping (Malécot *et al.*, 1998), which may also cause PS scrambling as well as cell death.

Table 5.2 Effect of pharmacological reagents on PS scrambling in SCA patients.

Pharmacological reagents	Phosphatidylserine exposure
EDTA	↓
GsMTx-4	↑
Ruthenium red	↑

6 KCl cotransporter and physiological modulators

6.0 Introduction

KCCs are expressed in a large variety of cells such as RBCs, tumour cells and hepatocytes, and have the potential to mediate regulatory volume decrease (RVD) following cell swelling (Lauf *et al.*, 1992; Shen *et al.*, 2003).

In sickle RBCs, abnormal KCC activation can induce the loss of intracellular potassium and chloride with water following osmotically via other channels (aquaporins), leading to RBC shrinkage, [HbS] elevation and HbS polymerisation acceleration, which in turn induce the red cells to sickle (Quarmyne *et al.*, 2011). In SCD patients, the details of KCC activation are complex and remain unclear, with various possible stimuli including the Mg^{2+} depletion (Jennings & Al-Rohil, 1990). Pharmacological evidence and molecular biology indicate that KCC is regulated by protein phosphorylation/ dephosphorylation, via cascades of regulatory protein kinases and phosphatases such as protein kinases Syk and Src or phosphatases PP1 and PP2A (Bize *et al.*, 2000). Overall, KCC activation has been concluded a net dehydration event, which is associated with solute loss and water dehydration.

Suspension in autologous plasma has previously been shown to inhibit markedly the activity of the KCC in RBCs from patients with SCD (Godart *et al.*, 1997).The cause appeared to be the presence of HCO_3^- / CO_2 buffer system (i.e. bicarbonate-buffered saline, BBS), when compared to artificial buffers such as MBS (MOPS-buffered saline), rather than some other component of plasma. Their finding prompted the authors of this earlier report to ask the question "Do HbSS erythrocytes lose KCl in physiological conditions?", thereby questioning the importance of KCC activity in mediating solute loss and dehydration of sickle cells.

The work of Godart *et al.*, however, only investigated the effect of plasma on RBCs swollen by dilution of their incubation solution with water. *In vivo*, such anisotonic swelling of red cells is an unlikely occurrence. The main physiological stimuli for KCC, urea (as concentrated in the renal medulla) or low pH (as encountered in active muscle beds) and Mg^{2+} depletion, were not tested. A key difference between anisotonic swelling as a stimulus, and those provided by low pH or urea, is their effect on ionic strength and $[Cl^-]$ - with a reduction of both by addition of water but not by the other

two modalities. In many cells, change in [Cl^-] has profound effects on cell behaviour including on volume regulatory systems such as KCC (Voets *et al.*, 1999). Furthermore, in RBCs from another species with physiologically high levels of KCl cotransport, namely horses, neither suspension in plasma nor BBS had any effect on KCC activity when using swelling or low pH as stimuli (Speake *et al.*, 1997). Urea was not tested although it is known to stimulate KCC activity in equine red cells (Speake *et al.*, 1997). These factors prompted a re-evaluation of the effect of plasma on RBC KCC activity.

In this chapter, a third model species in which red cell KCC activity has been much studied, sheep, was also utilised for comparison with HbSS RBCs: first, because HbSS RBCs appear to differ to findings with horse; and, second, because volume of plasma is a major limiting factor in these experiments due to the low volume of samples available from SCD patients whilst it is much easier to obtain large samples from sheep.

The hypothesis underlying the work is that swelling-stimulation of KCC in sickle cells is not representative of the effect of plasma on KCC activity more generally, so questioning the assertion of Godart *et al.* and re-emphasising a central importance of KCC activity to pathogenesis of SCD.

6.1 Results

6.2 Effect of external pH on oxygen-dependent KCl cotransport in HbSS RBCs

In this experiment, effect of external pH on oxygen-dependent KCl cotransport in HbSS RBCs was investigated in different incubation media: Cl-MBS, BBS and autologous plasma. As shown in Figure 6.1 (a), K^+ influxes were significantly greater in pH 7.0 incubation media, compared with pH 7.4 incubation media in PO_2 = 677 mmHg. Comparing K^+ influxes in pH 7.4 incubation media with pH 7.0 incubation media in PO_2 = 677 mmHg, K^+ influxes were relatively increased by about 35% ($p \leq 0.05$) in Cl-MBS; 69% ($p \leq 0.05$) in BBS and 93% ($p \leq 0.05$) in plasma.

In Figure 6.1 (b), K^+ influxes were also significantly greater in pH 7.0 incubation media, compared with pH 7.4 incubation media in PO_2 = 100 mmHg. Comparing K^+ influxes in pH 7.4 incubation media with pH 7.0 incubation media in PO_2 = 677 mmHg, K^+ influxes were relatively increased by about 24% ($p \leq 0.05$) in Cl-MBS; 70% ($p \leq 0.05$) in BBS and 125% ($p \leq 0.05$) in plasma.

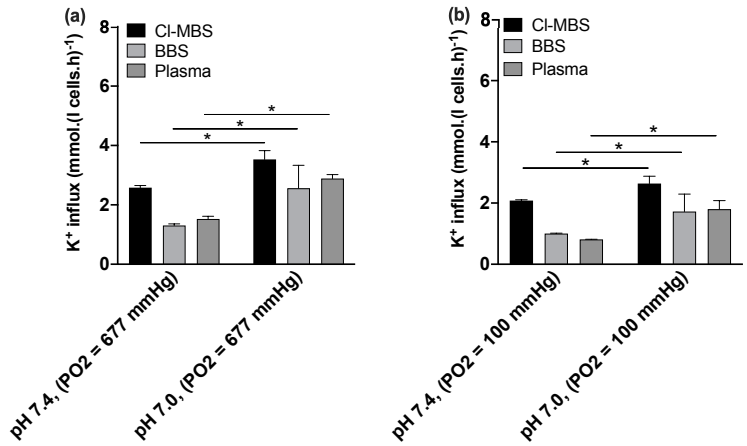

Figure 6.1 Effect of external pH on oxygen-dependent KCl cotransport in HbSS RBCs. HbSS RBCs (50% Hct) were equilibrated in Eschweiler tonometer for 15 min at desired oxygen tension (677 mmHg or 100 mmHg). (a) was incubated in PO_2 = 677 mmHg; (b) was incubated in PO_2 = 100 mmHg. Experimental incubation media pHs were pH 7.4 or pH 7.0, adjusted by utilising HNO_3 in MBS, but were gassed with PO_2=677 (100) mmHg/PCO_2=43 mmHg to obtain either pH 7.4 BBS or pH 7.4 plasma; PO_2=677 (100) mmHg/PCO_2=100 mmHg to obtain either pH 7.0 BBS or pH 7.0 plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min. All influxes were carried out in the presence of ouabain (100 µM) and bumetanide (10 µM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. Histograms represent means ± S.E.M., n = 3. * $p < 0.05$ by Student's t test.

6.3 Effect of urea on oxygen-dependent KCl cotransport in HbSS RBCs

In this experiment, the effect of urea on oxygen-dependent KCl cotransport in HbSS RBCs was investigated in different incubation media: Cl-MBS, BBS and autologous plasma. As shown in Figure 6.2 (a), K^+ influxes were significantly greater in incubation media in the presence of 750 mM urea, compared with incubation media in the absence of urea in PO_2 = 677 mmHg. Comparing K^+ influxes in incubation media in the presence of urea with incubation media in the absence of urea in PO_2 = 677 mmHg, K^+ influxes were relatively increased by about 59% ($p \leq 0.05$) in Cl-MBS; 50% ($p \leq 0.05$) in BBS and 25% ($p \leq 0.05$) in plasma.

In Figure 6.2 (b), K^+ influxes were significantly greater in incubation media in the presence of 750 mM urea, compared with incubation media in the absence of urea in PO_2 = 100 mmHg. Comparing K^+ influxes in incubation media in the presence of urea with incubation media in the absence of urea in PO_2 = 100 mmHg, K^+ influxes were relatively increased by about 92% ($p \leq 0.05$) in Cl-MBS; 71% ($p \leq 0.05$) in BBS and 30% ($p \leq 0.05$) in plasma.

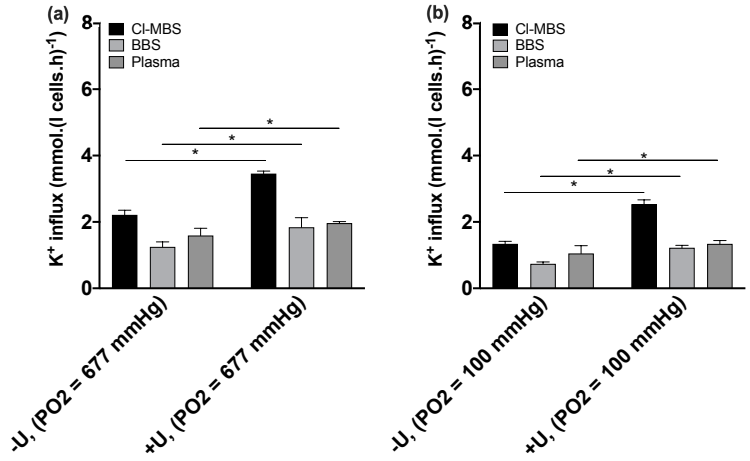

Figure 6.2 Effect of urea on oxygen-dependent K^+ influx in HbSS RBCs. HbSS RBCs (50% Hct) were equilibrated in Eschweiler tonometers for 15 min at an oxygen tension of 100 mmHg. Experimental pH was pH 7.4, adjusted utilising HNO_3 in N-MBS and Cl-MBS, but with PCO_2 = 43 mmHg in the case of BBS and autologous plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular $[K^+]$ of 7.5 mM) over 10 min, in which KCC activity was altered by addition of urea (750 mM). All influxes were carried out in the presence of ouabain (100 μM) and bumetanide (10 μM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. Herein, "-U" represents HbSS RBCs was incubated with incubation media (in the absence of urea); "+U" represents HbSS RBCs was incubated with incubation media (in the presence of 750 mM urea). Histograms represent means ± S.E.M., n = 3. * $p \leq 0.05$ by Student's t test.

6.4 Effect of cell volume on oxygen-dependent KCl cotransport in HbSS RBCs

In this experiment, effect of cell volume on oxygen-dependent KCl cotransport in HbSS RBCs was investigated in different incubation media: Cl-MBS, BBS and autologous plasma. As shown in Figure 6.3 (a), K^+ influxes were significantly greater in 15% swollen RBCs, compared with normal volume at PO_2 = 677 mmHg. The relative increases were about 115% ($p \leq 0.01$) in Cl-MBS; 35% ($p \leq 0.05$) in BBS and 61% ($p \leq 0.05$) in plasma.

In Figure 6.3 (b), K^+ influxes were also significantly greater in 15% swollen RBCs at PO_2 = 100 mmHg. The relative increases were about 171% ($p \leq 0.01$) in Cl-MBS; 108% ($p \leq 0.05$) in BBS and 100% ($p \leq 0.05$) in plasma.

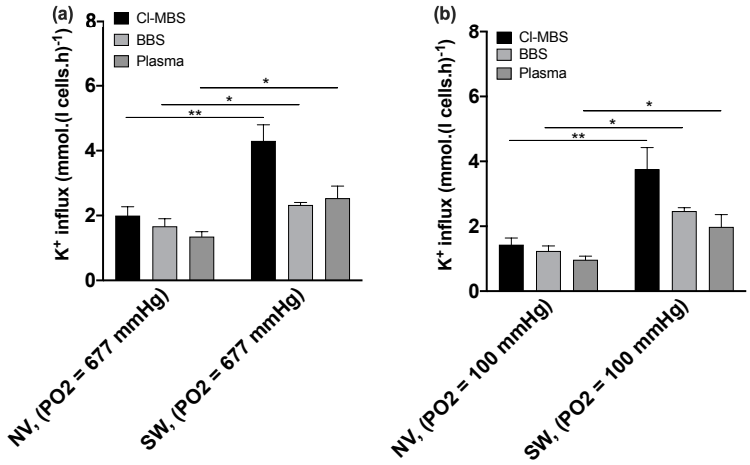

Figure 6.3 Effect of cell volume on oxygen-dependent K^+ influxes in HbSS RBCs. HbSS RBCs (50% Hct) were equilibrated in Eschweiler tonometer for 15 min at an O_2 tensions of: (a) 677 mmHg or (b) 100 mmHg. Experimental pH was pH 7.4, adjusted using HNO_3 in Cl-MBS, but with PCO_2 = 43 mmHg in the case of plasma and BBS. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min, in which RBC volume was altered anisotonically through addition of water. All influxes were carried out in the presence of ouabain (100 μM) and bumetanide (10 μM) to inhibit K^+ influx through the Na^+/K^+ pump and Na^+-K^+-Cl^- cotransporter (NKCC), respectively. "NV" represents red cells was in normal volume. "SW" represents the changes of flux induced by 15% swollen of HbSS RBCs under anisotonically condition. Histograms represent means ± S.E.M., n = 3. * $p \leq 0.05$; ** $p \leq 0.01$ by Student's t test.

6.5 Effect of incubation media on pH-dependent KCl cotransport in HbSS RBCs

In human bodies, the external pH of RBCs may change in active muscle beds from the normal pH value of pH 7.4 to pH 7.0 or 6.5 *in vivo* (Nielsen et al., 2002; Richardson, 2000). In this experiment, the effects of incubation media on pH-dependent KCl cotransport in HbSS RBCs were investigated. The pH values of incubation media were adjusted by utilising HNO_3 in Cl-MBS, but the pH value of BBS/plasma was altered by gassing with different partial pressures of CO_2 (PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg for pH 7.4; PO_2=100 mmHg/PN_2=513 mmHg/PCO_2=100 mmHg for pH 7.0; PO_2=100 mmHg/PN_2=292 mmHg/PCO_2=321 mmHg for pH 6.5). In all cases, osmolality, pH, extracellular K^+ concentration and O_2 tension were all carefully controlled.

As shown in Figure 6.4, K^+ influxes were significantly greater at pH 7.0 incubation compared with pH 7.4. In Cl-MBS, K^+ influxes were relatively increased by about 68% ($p \leq 0.05$), in BBS by about 58% ($p \leq 0.05$) and in plasma by about 17% ($p \leq 0.05$). The results show that KCC in HbSS RBCs is pH-dependent under isotonic conditions. Low pH will induce RBCs to swell, however, and part of the acid-induced stimulation of KCC may be mediated by this mechanism.

To further investigate the effect of incubation media on pH-dependent KCl cotransport in HbSS RBCs, the right-hand panel of Figure 6.4 gives the pH-dependent components of K^+ influx (= influx in pH 7.0 incubation media – influx in pH 7.4 incubation media). The changes of K^+ influxes (pH-dependent fluxes, pHDF) induced by low pH value of incubation media were significantly greater in Cl-MBS, followed by BBS and then plasma. Herein, comparing influxes changes in BBS with influxes changes in Cl-MBS, influxes changes in BBS was 62% lower ($p \leq 0.05$), whilst they were 44% decreased ($p \leq 0.05$) comparing plasma with BBS, and 79% lower ($p \leq 0.05$) comparing plasma with that in Cl-MBS.

To summarise, the results confirmed that when the external pH values of incubation media were altered between pH 7.4 and pH 7.0, the K^+ influx in plasma was less than in either Cl-MBS or in BBS, implying that autologous plasma had an inhibitory effect on pH-dependent KCl cotransport in HbSS RBCs.

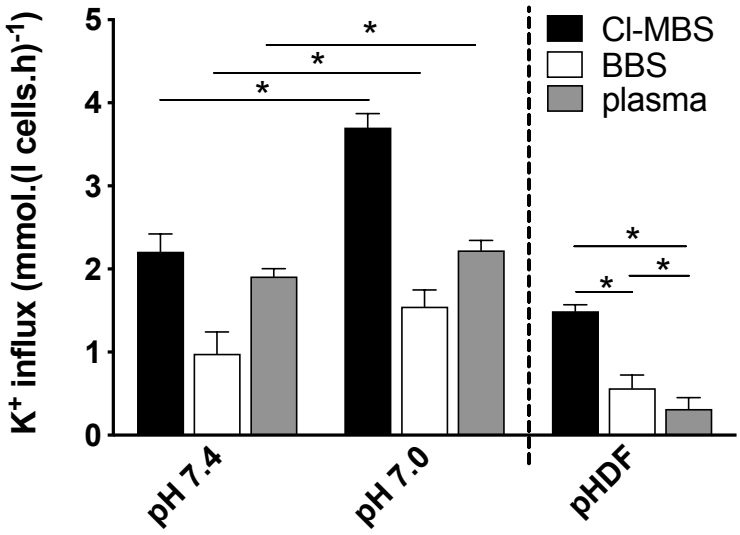

Figure 6.4 The effect of external pH on K^+ influxes measured in Cl-MBS, BBS and plasma in HbSS RBCs volume under isotonic condition. HbSS RBCs (50% Hct) were equilibrated in Eschweiler tonometers for 15 min at desired oxygen tension. Experimental incubation media pH(s) were pH 7.4 and pH 7.0, adjusted by utilising HNO_3 in Cl-MBS, but were gassed with PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg to obtain either pH 7.4 BBS or pH 7.4 plasma and PO_2=100 mmHg/PN_2=513 mmHg/PCO_2=100 mmHg to obtain either pH 7.0 BBS or pH 7.0 plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min. All influxes were carried out in the presence of ouabain (100 µM) and bumetanide (10 µM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. Histograms represent means ± S.E.M., n = 3. * $p \leq 0.05$, by Student's t test.

6.6 Effect of incubation media on urea-stimulated KCl cotransport in HbSS RBCs

In this experiment, the effects of incubation media on urea-stimulated K^+ influxes were investigated. In all cases, osmolality, pH, extracellular [K^+] and O_2 tension were all carefully controlled. In HbSS RBCs, in the presence of bumetanide and ouabain, urea-stimulated K^+ transport is mediated solely by KCC.

Figure 6.5 shows the effect of autologous plasma in comparison with Cl-MBS and BBS on urea-stimulated K^+ influx in HbSS RBCs. The two left-hand panels show K^+ influx in RBCs incubated in the absence/ presence of urea (750 mM). In all cases, K^+ influx was increased by addition of urea, with relative increase in Cl-MBS, BBS, and plasma being 38% ($p \leq 0.05$), 59% ($p \leq 0.05$), 45% ($p \leq 0.05$), respectively.

The right-hand panel of Figure 6.5 gives the urea-stimulated components of K^+ influx (= influx in urea-treated RBCs – influx in urea-untreated RBCs). There was no significant effect of incubation media on urea-stimulated K^+ influxes in HbSS RBCs.

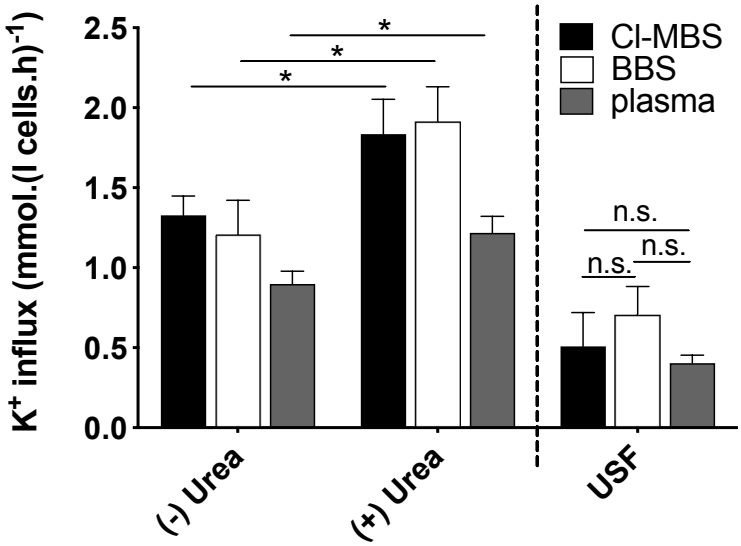

Figure 6.5 The effect of urea on K^+ influx in HbSS RBCs. HbSS RBCs (50% Hct) were equilibrated in Eschweiler tonometer for 15 min at an oxygen tension of 100 mmHg. Experimental pH was pH 7.4, adjusted utilising HNO_3 in N-MBS and Cl-MBS, but with PCO_2 = 43 mmHg in the case of BBS and autologous plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min, in which KCC activity was altered by addition of urea (750 mM). All influxes were carried out in the presence of ouabain (100 µM) and bumetanide (10 µM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. USF represents the changes of flux induced by high urea concentration in incubation media. Histograms represent means ± S.E.M., n = 4. * $p \leq 0.05$; n.s. indicates "not significant" by Student's t test.

6.7 Effect of incubation media on volume-sensitive KCl cotransport in HbSS RBCs

In this series of experiments, volume-sensitive K^+ influxes were investigated. In all cases, osmolality, pH, extracellular [K^+] and O_2 tension were all carefully controlled. In HbSS RBCs, in the presence of bumetanide, volume-sensitive K^+ transport is mediated solely by KCC.

Figure 6.6 shows the effect of autologous plasma in comparison with Cl-MBS and BBS on volume-sensitive K^+ influx in HbSS RBCs. The two left-hand panels show K^+ influx in RBCs shrunken or swollen anisotonically by 15% through addition of hypertonic sucrose or water to the incubation media. In all cases, K^+ influx was increased on swelling, with relative increases in Cl-MBS, BBS and plasma being 543% ($p \leq 0.01$), 232% ($p \leq 0.01$) and 147% ($p \leq 0.05$), respectively. There was no significant effect of incubation media on K^+ influxes in shrunken RBCs. Influxes in swollen RBCs, however, showed a significant inhibitory effect of BBS and plasma compared to MBS.

The right-hand panel of Figure 6.6 gives the volume-sensitive components of K^+ influx (= influx in swollen RBCs – influx in shrunken RBCs). Volume-sensitive K^+ influxes were significantly greater in Cl-MBS, followed by BBS and then plasma. Comparing Cl-MBS with BBS, K^+ influxes in Cl-MBS were 41% higher ($p \leq 0.05$) than that in BBS, whilst there were 69% higher ($p \leq 0.01$) when influx in Cl-MBS was compared with plasma, and 48% higher ($p \leq 0.05$) when comparing BBS with plasma.

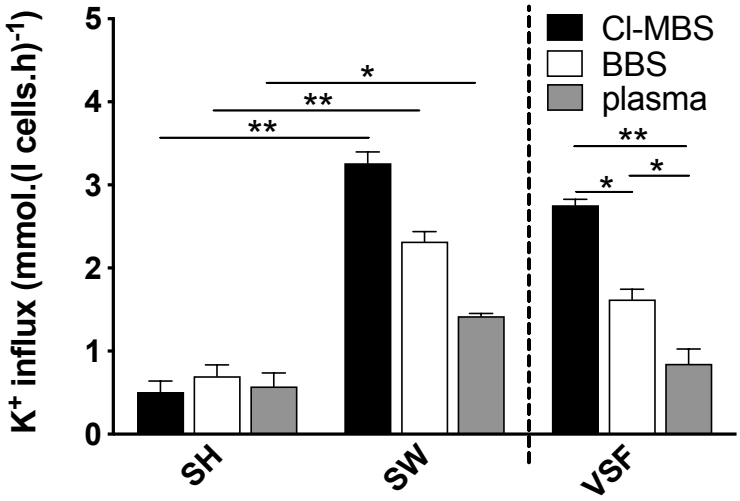

Figure 6.6 The effect of incubation media on volume-sensitive K^+ influxes in HbSS RBCs. HbSS RBCs (50% Hct) were equilibrated in Eschweiler tonometers for 15 min at an O_2 tension of 100 mmHg. Experimental pH was pH 7.4, adjusted using HNO_3 in Cl-MBS, but with PCO_2 = 43 mmHg in the case of plasma and BBS. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min, in which RBC volume was altered anisotonically through addition of hypertonic sucrose or water. All influxes were carried out in the presence of ouabain (100 µM) and bumetanide (10 µM) to inhibit K^+ influx through the Na^+/K^+ pump and Na^+-K^+-Cl^- cotransporter (NKCC), respectively. VSF represents the changes of flux induced by 15% shrunken/swollen of HbSS RBCs under anisotonic conditions. Histograms represent means ± S.E.M., n = 4. * $p \leq 0.05$; ** $p \leq 0.01$ by Student's t test.

6.8 Effect of incubation media on pH-dependent KCl cotransport in LK sheep RBCs

In this experiment, the effects of incubation media on pH-dependent KCl cotransport in LK sheep RBCs were investigated. The pH values of incubation media were adjusted by utilising HNO_3 in Cl-MBS, but the pH value of BBS/plasma was altered by gassing with different partial pressures of CO_2 (PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg for pH 7.4; PO_2=100 mmHg/PN_2=513 mmHg/PCO_2=100 mmHg for pH 7.0; PO_2=100 mmHg/PN_2=292 mmHg/PCO_2=321 mmHg for pH 6.5). In all cases, osmolality, pH, extracellular K^+ concentration and O_2 tension were all carefully controlled.

As shown in Figure 6.7, K^+ influxes were significantly greater at pH 7.0 compared with pH 6.5 or pH 7.4. In Cl-MBS, comparing K^+ influxes at pH 7.4 with pH 7.0 and pH 6.5, K^+ influxes were relatively increased by about 103% ($p \leq 0.05$) and 45% ($p \leq 0.05$). In BBS, comparing K^+ influxes at pH 7.4 with pH 7.0 BBS and pH 6.5, K^+ influxes were relatively increased by about 44% ($p \leq 0.05$) and 25% ($p \leq 0.05$). In plasma, comparing K^+ influxes at pH 7.4 with pH 7.0 and pH 6.5, the K^+ influxes were relatively increased by about 38% ($p \leq 0.05$) and 31% ($p \leq 0.05$). The results show that KCC in LK sheep RBCs is pH-dependent under isotonic conditions.

To further investigate the effect of incubation media on pH-dependent KCl cotransport in LK sheep RBCs, Figure 6.8 gives the pH-dependent components of K^+ influx (= influx in pH 7.0 incubation media – influx in pH 7.4 incubation media). The changes of K^+ influxes (pHDF) induced by low pH value of incubation media were significantly greater in Cl-MBS, followed by BBS and then plasma. Compared with Cl-MBS (pHDF1), influxes in BBS (pHDF2) were reduced by 37% ($p \leq 0.01$), whilst comparing Cl-MBS (pHDF1) with plasma (pHDF3) they were 26% reduced ($p \leq 0.05$).

Figure 6.9 compares inflxues at pH 7.4 with pH 6.5 giving the pH-dependent components of K^+ influx (= influx in pH 6.5 incubation media – influx in pH 7.4 incubation media, pHDF). Changes were still significantly greater in Cl-MBS, followed by BBS and then plasma: 17 % lower in BBS cf Cl-MBS (pHDF4); 46 % lower in plasma cf BBS (pHDF5); and 55 % lower comparing plasma with Cl-MBS (pHDF6).

To summarise, the results confirmed that when the external pH values of incubation media were altered from pH 7.4 to either pH 7.0 or pH 6.5, the K^+ influx in plasma

was less than either in Cl-MBS or in BBS, implying autologous plasma had a larger inhibitory effect on pH-dependent KCl cotransport in LK sheep RBCs. However, at pH 7.0 when KCC activity was greatest, there was no differences observed in any of the incubation media, unlike the case with HbSS RBCs.

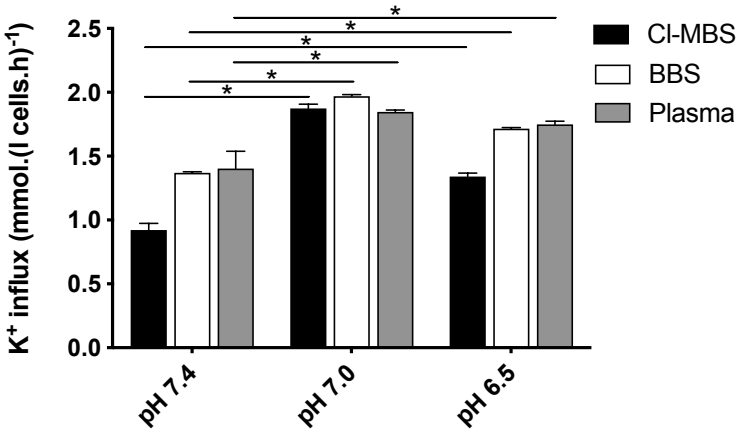

Figure 6.7 The effect of external pH on K^+ influxes measured in Cl-MBS, BBS and plasma in LK sheep RBCs volume under isotonic conditions. Sheep RBCs (50% Hct) were equilibrated in Eschweiler tonometers for 15 min at the desired oxygen tension. Experimental incubation media pHs were pH 7.4, pH 7.0 and pH 6.5, adjusted by utilising HNO_3 in Cl-MBS, but were gassed with PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg to obtain either pH 7.4 BBS or pH 7.4 plasma; PO_2=100 mmHg/PN_2=513 mmHg/PCO_2=100 mmHg to obtain either pH 7.0 BBS or pH 7.0 plasma; and PO_2=100 mmHg/PN_2=292 mmHg/PCO_2=321 mmHg to obtain either pH 6.5 BBS or pH 6.5 plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min. All influxes were carried out in the presence of ouabain (100 μM) and bumetanide (10 μM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. Histograms represent means ± S.E.M., n = 3. * $p \leq 0.05$ by Student's t test.

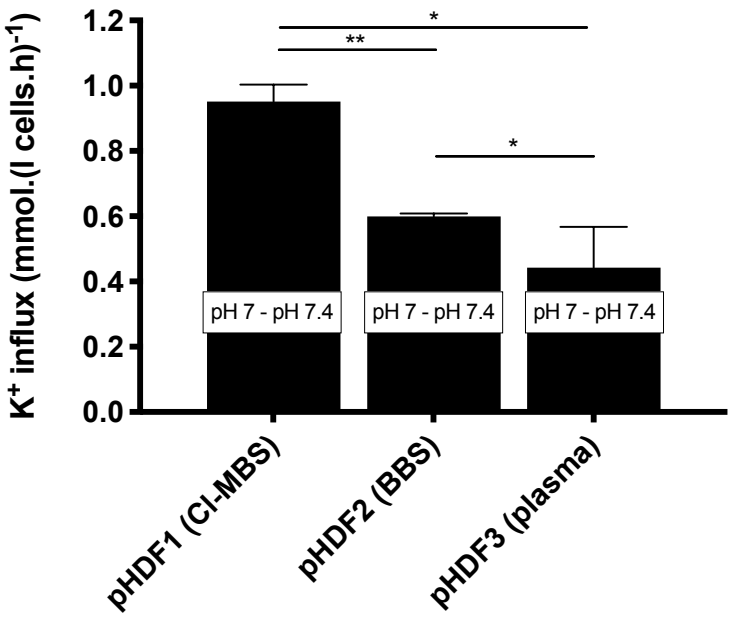

Figure 6.8 The effect of incubation media on KCl cotransport in LK sheep RBCs (the pH value of incubation media was altered from pH 7.4 to pH 7.0 under isotonic condition). Experimental incubation media pHs were pH 7.4 and pH 7.0, adjusted by utilising HNO_3 in Cl-MBS, but were gassed with PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg to obtain either pH 7.4 BBS or pH 7.4 plasma; PO_2=100 mmHg/PN_2=513 mmHg/PCO_2=100 mmHg to obtain either pH 7.0 BBS or pH 7.0 plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min. All influxes were carried out in the presence of ouabain (100 μM) and bumetanide (10 μM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. pHDF represents the changes of flux induced by lower pH of incubation media. Histograms represent means ± S.E.M., n = 3. * $p \leq 0.05$; * $p \leq 0.01$ by Student's t test.

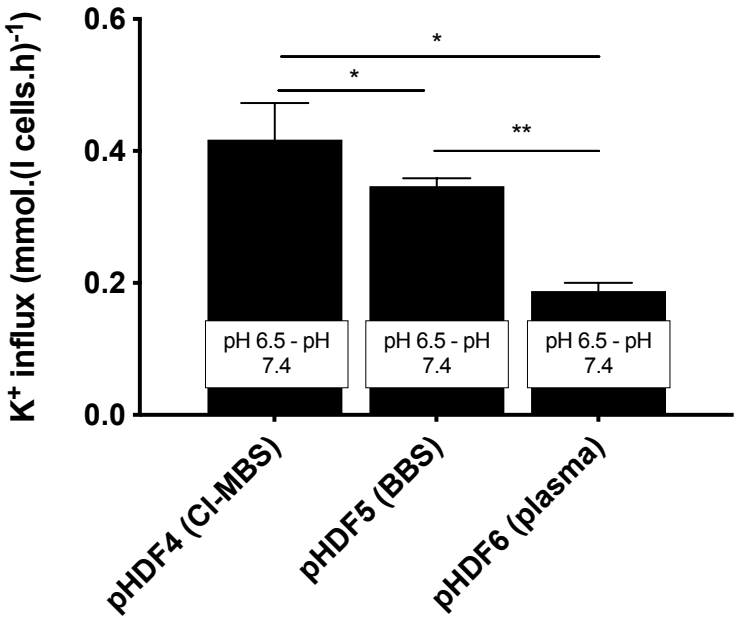

Figure 6.9 The effect of incubation media on KCl cotransport in LK sheep RBCs when the pH value of incubation media was altered from pH 7.4 to pH 6.5 under isotonic condition. Experimental incubation media pHs were pH 7.4 and pH 6.5, adjusted by utilising HNO_3 in Cl-MBS, but were gassed with PO_2=100 mmHg/PN_2=570 mmHg/PCO_2=43 mmHg to obtain either pH 7.4 BBS or pH 7.4 plasma; and PO_2=100 mmHg/PN_2=292 mmHg/PCO_2=321 mmHg to obtain either pH 6.5 BBS or pH 6.5 plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min. All influxes were carried out in the presence of ouabain (100 μM) and bumetanide (10 μM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. pHDF represents the changes of flux induced by lower pH of incubation media. Histograms represent means ± S.E.M., n = 3. * $p \leq 0.05$; * $p \leq 0.01$ by Student's t test.

6.9 Effect of incubation media on urea-stimulated KCl cotransport in LK sheep RBCs

In this experiment, the effects of incubation media on urea-stimulated K^+ influxes were investigated. In all cases, osmolality, pH, extracellular [K^+] and O_2 tension were all carefully controlled. In LK sheep, in the presence of bumetanide and ouabain, urea-stimulated K^+ transport is mediated solely by KCC.

Figure 6.10 shows the effect of autologous plasma in comparison with Cl-MBS and BBS on urea-stimulated K^+ influx in LK sheep RBCs. The two left-hand panels show K^+ influx in RBCs incubated in the absence/ presence of urea (750 mM). In all cases, K^+ influx was increased by addition of urea, with relative increase in Cl-MBS, BBS, and plasma being 184% ($p \leq 0.05$), 128% ($p \leq 0.05$), 117% ($p \leq 0.05$), respectively.

The right-hand panel of Figure 6.10 gives the urea-stimulated components of K^+ influx (= influx in urea-treated RBCs – influx in urea-untreated RBCs). There was no significant effect of incubation media on urea-stimulated K^+ influxes in LK sheep RBCs.

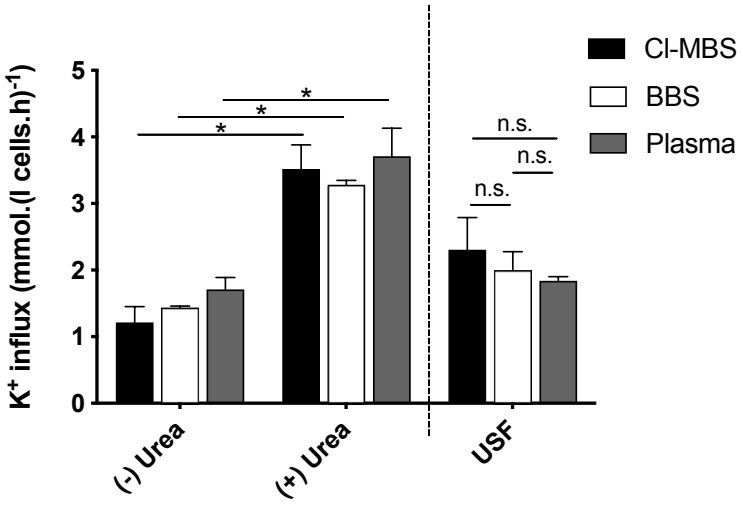

Figure 6.10 The effect of urea on K^+ influx in LK sheep RBCs. Sheep RBCs (50% Hct) were equilibrated in Eschweiler tonometer for 15 min at an oxygen tension of 100 mmHg. Experimental pH was pH 7.4, adjusted utilising HNO_3 in N-MBS and Cl-MBS, but with PCO_2 = 43 mmHg in the case of BBS and autologous plasma. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min, in which KCC activity was altered by addition of urea (750 mM). All influxes were carried out in the presence of ouabain (100 µM) and bumetanide (10 µM) to inhibit K^+ influx through the Na^+/K^+ pump and NKCC, respectively. USF represents the changes of flux induced by high urea concentration in incubation media. Histograms represent means ± S.E.M., n = 4. * $p \leq 0.05$; n.s. indicates "not significant" by Student's t test.

6.10 Effect of incubation media on volume-sensitive KCl cotransport in LK sheep RBCs

In this series of experiments, volume-sensitive K^+ influxes were investigated. In all cases, osmolality, pH, extracellular [K^+] and O_2 tension were all carefully controlled. In LK sheep RBCs, in the presence of bumetanide, volume-sensitive K^+ transport is mediated solely by KCC.

Figure 6.11 shows the effect of autologous plasma in comparison with Cl-MBS and BBS on volume-sensitive K^+ influx in LK sheep RBCs. The two left-hand panels show K^+ influx in RBCs shrunken or swollen anisotonically by 15% through addition of hypertonic sucrose or water to the incubation media. In all cases, K^+ influx was increased on swelling, with relative increases in Cl-MBS, BBS and plasma being 78% ($p \leq 0.05$), 66% ($p \leq 0.01$) and 49% ($p \leq 0.01$), respectively. There was no significant effect of incubation media on K^+ influxes in shrunken RBCs. Influxes in swollen RBCs, however, showed a significant effect of incubation media on K^+ influxes.

The right-hand panel of Figure 6.11 gives the volume-sensitive components of K^+ influx (= influx in swollen RBCs – influx in shrunken cells). Volume-sensitive K^+ influxes were significantly greater in Cl-MBS, followed by BBS and then plasma. Comparing Cl-MBS with BBS, K^+ influxes in Cl-MBS were 37% higher ($p \leq 0.05$) than that in BBS, whilst there were 98% higher ($p \leq 0.01$) when influx in Cl-MBS was compared with plasma, and 45% higher ($p \leq 0.05$) when comparing BBS with plasma.

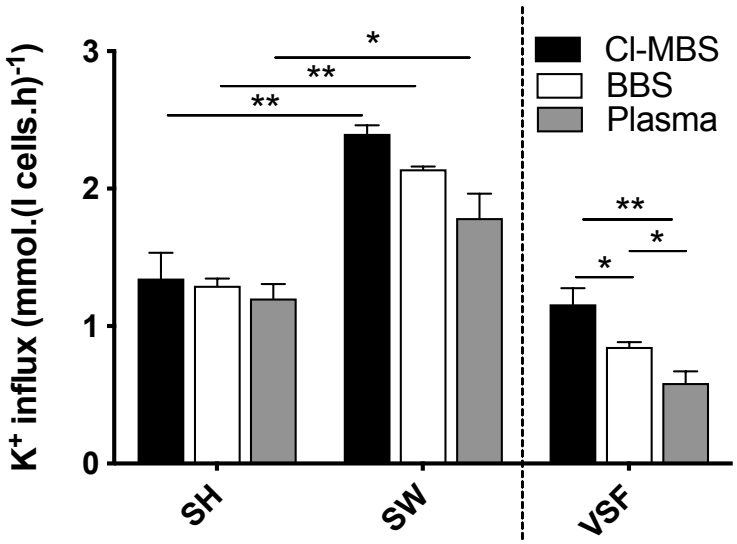

Figure 6.11 The effect of incubation media on volume-sensitive K^+ influxes in LK sheep RBCs. Sheep RBCs (50% Hct) were equilibrated in Eschweiler tonometers for 15 min at an O_2 tension of 100 mmHg. Experimental pH was pH 7.4, adjusted using HNO_3 in Cl-MBS, but with PCO_2 = 43 mmHg in the case of plasma and BBS. RBC aliquots were then diluted 10-fold into test tubes pre-equilibrated with the same gas mixtures for measurement of K^+ influx (extracellular [K^+] of 7.5 mM) over 10 min, in which RBC volume was altered anisotonically through addition of hypertonic sucrose or water. All influxes were carried out in the presence of ouabain (100 μM) and bumetanide (10 μM) to inhibit K^+ influx through the Na^+/K^+ pump and Na^+-K^+-Cl^- cotransporter (NKCC), respectively. VSF represents the changes of flux induced by 15% shrunken/swollen of LK sheep RBCs under anisotonic conditions. Histograms represent means ± S.E.M., n = 4. * $p \leq 0.05$; ** $p \leq 0.01$ by Student's t test.

6.11 Discussion and conclusion

KCC activity is sensitive to various physiological stimuli including oxygen tension, pH, urea, bicarbonate and volume in RBCs from many vertebrates including sheep, , human and horse (Ellory *et al.*, 1991; Hoffmann & Dunham, 1995; Godart *et al.*, 1997; Gibson *et al.*, 2000; Joiner *et al.*, 2004). In order to investigate further the role of these physiological stimuli roles in KCC modulation, LK sheep red cells were utilised in these series of experiments, as KCC in LK sheep RBCs shares similar properties with that in equine RBCs, human HbSS RBCs and human umbilical cord RBCs (Lauf *et al.*, 1992; Speake *et al.*, 1997; Gibson *et al.*, 2001). Findings in LK sheep RBCs were also compared with those in human HbSS RBCs.

6.11.1 Comparison of the KCl cotransporter in LK sheep RBC with that in human HbSS RBCs, equine RBCs and human umbilical cord (HbF) RBCs

A previous study carried out by Godart *et al*. on HbSS RBCs only investigated the effect of autologous plasma under anisotonic conditions, notably hypotonic swelling, which is an unlikely occurrence *in vivo*. In this chapter, the other two main physiological stimuli for KCC *in vivo*, urea and low pH, were examined as well, as they will be more significant for KCC function in the circulation.

Table 6.1 summarises the characteristics of KCC in RBCs from the different species or stage of maturity. It can be seen that KCC activities were increased when cells became swollen. Also, urea and low pH can increase KCC activity in LK sheep RBCs, equine RBCs, human HbSS RBCs and HbF RBCs.

To summarise, the previous and present studies suggest that LK sheep RBCs share broadly similar properties with human HbSS RBCs, equine RBCs and immature human RBCs, in which low pH, urea and anisotonic swelling are three key physiological stimuli for KCC activation.

Table 6.1. Comparison of KCl cotransporter in LK sheep RBCs with that in human HbSS RBCs, equine RBCs, and human umbilical cord RBCs. All RBCs were incubated in MBS at 37 °C. KCC activity in equine RBCs, human RBCs and HbF RBCs were taken from the following studies: equine: Gibson *et al.*, 1993; Gibson *et al.*, 1995; Speake and Gibson, 1997; HbSS: Hannemann *et al.*, 2015 and HbF: Gibson *et al.*, 2001. "SH" represent 10% shrinkage of red cell volume; "SW" represents 10% swollen of red cell volume. DIOA was added at a concentration of 50 μM. N.D. indicates that "no data" was presented for these stimuli or inhibitors; n.s. indicates "not significant" by Student's t test.

	The influx changes in LK sheep RBCs (%)	**The influx changes in HbSS RBCs (%)**	**The influx changes in equine RBCs (%)**	**The influx changes in HbF RBCs (%)**
pH 7.4 Cl-MBS -> pH 7.0 Cl-MBS	+110%	+300%	+220%	+275%
pH 7.4 Cl-MBS -> pH 6.5 Cl-MBS	+45%	+75%	+270%	N.D.
Urea stimulation	+184%	+152%	+860%	+700%
SH -> SW in Cl-MBS	+78%	+186%	+15%	+600%

6.11.2 Variables affecting KCC activity: control experiments on pH, oxygen tension, temperature, extracellular [K^+] and osmolality

Technically, the pH value of BBS/plasma is more difficult to alter than that in salines buffered with artificial reagents, as it is determined by the partial pressures of CO_2 and the concentration of bicarbonate. pH, as an important physiological stimulus *in vivo*, plays an important role in modulating KCC activity – for example, low pH is encountered in active muscle beds. Hence, uniquely in this study, a Wösthoff gas mixing pump was utilised to control accurately the partial pressures of CO_2 over the physiological range of extracellular pHs.

According to Campbell and Gibson in 1998, PO_2 values over the physiological range can also affect the activity of KCC, presenting a sigmoidal relationship, which is probably altered by the O_2 saturation of Hb (Campbell & Gibson, 1998). Hence, in this study, both blood samples (HbSS RBCs and LK sheep RBCs) were also gassed with desired partial pressures of oxygen to control the oxygen-dependent component of KCC to remove this potential stimulus of variability.

Evidence shows that altered temperature can perturb KCC activity by affecting the action of regulatory protein kinases/phosphatases, such as activation of phosphatases at high temperature (Khan & Ellory, 2000). Hence, all experiments were carried out in the incubation media at 37 °C to remove this stimulus of variability.

Variations in extracellular [K^+] are also associated with changes in KCC activity. In RBCs, KCC has an affinity of about 7.5 mM extracellular [K^+], also any variation in [K^+] will alter the effective specific activity of $^{86}Rb^+$. Hence, before carrying out the experiments, the extracellular plasma [K^+] of LK sheep were measured. [K^+] in plasma and artificial salines were all adjusted to 7.5 mM (Brugnara *et al.*, 1986; Brugnara & Tosteson, 1987). Also, RBC [K^+] was checked to ensure that all experiment samples were from LK sheep.

KCC has been well studied as a volume-sensitive transport system. Therefore, to ascertain that the RBC volume was not significantly changed by incubation in different incubation media, control experiments measuring the water content of RBCs were also carried out (Brugnara *et al.*, 1986; Brugnara & Tosteson, 1987).

Hence, the effects of any variations in pH, oxygen tension, temperature, extracellular [K^+] and osmolality on KCC activity in LK sheep RBCs can be ignored in these experiments.

6.11.3 Effect of autologous plasma

In section 6.7 and section 6.10, the effect of incubation media on volume-sensitive K^+ influx in HbSS RBCs and LK sheep RBCs was investigated, although the effects of plasma on volume-sensitive K^+ influx in sickle cells have been studied in HbSS red cells previously (Godart *et al.*, 1997). In Figure 6.11, substitution of MBS with plasma significantly decreased the volume-sensitive K^+ influx in LK sheep RBCs, and this effect was observed in swollen RBCs where KCl cotransporter was activated. The result suggests that sheep plasma has an inhibitory effect on volume-sensitive KCl cotransport in LK sheep RBCs, similar to that observed by Godart *et al.* (1997) and the result observed in Figure 6.6 on HbSS RBCs confirmed these findings. Moreover, comparing volume-sensitive KCl cotransport in different species' RBCs (HbSS RBCs and LK sheep), substitution of BBS with plasma decreased the fluxes by about 31% and 0% on volume-sensitive K^+ flux in LK sheep RBCs and HbSS RBCs (Table 6.2),

which is consistent with the inhibitory substances in plasma being the bicarbonate/CO_2 buffer. Tables below (Table 6.2, Table 6.3 and Table 6.4) summarise the effect of incubation media on volume-sensitive KCl cotransport in LK sheep RBCs and HbSS RBCs. In the experiment carried out by Godart *et al.* in 1997, they utilised 5% CO_2 and 25 mM bicarbonate in their experiments, which gives a pH value of 7.5 rather than 7.4 (Godart *et al.*, 1997). Small changes in pH and cell volume at around pH 7.4 have substantial effects on KCC activity in HbSS RBCs (Brugnara *et al.*, 1986). It may be that the part of the inhibitory effect of plasma observed in their work is due to these factors.

Table 6.2 Effect of incubation media on volume-sensitive KCl cotransport in LK sheep RBCs and HbSS RBCs at pH 7.4, 37 °C (Godart *et al.*, 1997).

	The influx changes of volume-sensitive KCl cotransport in LK sheep RBCs (%)	**The influx changes of volume-sensitive KCl cotransport in human HbSS RBCs (%)**
Cl-MBS -> BBS	-27%	-70%
Cl-MBS -> plasma	-50%	-77%
BBS -> plasma	-31%	0%

Table 6.3 Effect of Cl-MBS and BBS on KCC in shrunken (SH) and swollen (SW) LK sheep RBCs and HbSS RBCs at pH 7.4, 37 °C (Godart *et al.*, 1997).

	The effect of incubation media on KCl cotransport in LK sheep RBCs (%)	**The effect of incubation media on KCl cotransport in human HbSS RBCs (%)**
SH Cl-MBS -> SH BBS	-4%	-4%
SW Cl-MBS -> SW BBS	-11%	-59%

Table 6.4 Effect of Cl-MBS and plasma on KCC in shrunken (SH) and swollen (SW) LK sheep RBCs and HbSS RBCs at pH 7.4, 37 °C (Godart *et al.*, 1997).

	The effect of incubation media on KCl cotransport in LK sheep RBCs (%)	**The effect of incubation media on KCl cotransport in human HbSS RBCs (%)**
SH Cl-MBS -> SH plasma	-11%	-7%
SW Cl-MBS -> SW plasma	-25%	-39%

In section 6.6 and section 6.9, the effect of incubation media on urea-sensitive K^+ influx in HbSS RBCs and LK sheep RBCs was studied. To obtain high KCC activities in the effect of incubation media, high urea concentrations (750 mM) were utilised (Dunham, 1995). The present study (Figure 6.5 and Figure 6.10) demonstrates that in the absence/presence of urea (high concentration), there were no significant effect of incubation media on the activity of KCl cotransport both in HbSS RBCs and LK sheep RBCs when comparing either Cl-MBS, BBS, or plasma (Table 6.5). The cause might be that high urea concentrations mimic RBC swelling through effects on macromolecular crowding, which in turn might inhibit regulatory enzymes directly (Speake *et al.*, 1997). It is postulated that under these conditions, when KCC activity is maximally activated,

that no differences were observed when comparing the effect of different incubation media.

Table 6.5 Effect of incubation media on urea-stimulated KCl cotransport in LK sheep RBCs and HbSS RBCs at pH 7.4, 37 °C.

	The influx changes of urea-stimulated KCl cotransport in LK sheep RBCs (%)	**The influx changes of urea-stimulated KCl cotransport in human HbSS RBCs (%)**
Cl-MBS -> BBS	n.s.	n.s.
Cl-MBS -> plasma	n.s.	n.s.
BBS -> plasma	n.s.	n.s.

n.s. represent "not significant".

In section 6.5 and section 6.8, the effect of incubation media on pH-dependent K^+ influx in HbSS RBCs and LK sheep RBCs was investigated. Table 6.6 summarises the effect of incubation media on pH-dependent KCl cotransport in LK sheep RBCs and HbSS RBCs at pH 7.4 and pH 7.0. In Figure 6.4 and Figure 6.8, plasma had an inhibitory effect on pH-dependent KCl cotransport in HbSS red cells and LK sheep RBCs, compared with Cl-MBS.

Table 6.6 Effect of incubation media on pH-dependent KCl cotransport in LK sheep RBCs and HbSS RBCs at pH 7.4 and pH 7.0, 37 °C.

	The influx changes of pH-dependent KCl cotransport in LK sheep RBCs (%)	**The influx changes of pH-dependent KCl cotransport in human HbSS RBCs (%)**
Cl-MBS -> BBS	-39%	-62%
Cl-MBS -> plasma	-54%	-79%
BBS -> plasma	-27%	-44%

In conclusion, findings suggested that KCC activity could be altered by O_2, external pH, urea, cell volume, buffer salines. This chapter further compared the effect of these stimuli in different buffer systems in both HbSS RBCs and sheep RBCs. Unlike the situation in horse RBCs, bicarbonate / CO_2 buffers had some inhibitory effects on some, but not all stimuli. The relative inhibitory effects, however, were considerably smaller than those described by Godart *et al.*, 1997, particularly for urea-stimulated and pH-stimulated fluxes, suggesting that KCC activity *in vivo* could contribute to RBC dehydration.

7 KCl cotransport and pharmacological modulators

7.0 Introduction

In human red cells, the major significance of KCC is probably pathological in SCA patients as its increased and abnormal activity will result in solute loss, RBC shrinkage and hence elevated [HbS], thus promoting HbS polymerisation and sickling. It may therefore represent an important early stage in pathogenesis. Considerable effort has therefore been expended on understanding the underlying mechanisms and in the design of potential pharmacological inhibitors (Gibson *et al.*, 2015).

As outlined previously, protein phosphorylation is a key component in regulation of KCC activity, in both normal and sickle RBCs from humans and across vertebrate species (Jennings & Al-Rohil, 1990; Jennings & Schulz, 1991; Cossins *et al.*, 1994; Flatman *et al.*, 1996). Net dephosphorylation of the transporter, or a regulatory protein, is associated with higher KCC activity and net phosphorylation with reduced activity (Cossins *et al.*, 1994). Notwithstanding, most work has been carried out using more or less specific pharmacological inhibitors (staurosporine, genistein, *N*-ethylmaleimide, calyculin A), and the identity of the specific enzymes involved remains unclear (Cossins *et al.*, 1994).

An important breakthrough came when it was found that some cases of hypertension were caused by mutations in the WNK kinases (Wilson *et al.*, 2001). It was then shown that some cation chloride cotransporters (CCCs) were regulated by two Ste20 group kinases, the oxidative stress response kinase 1 (OSR1) and the SPS1-related proline/alanine-rich kinase (SPAK or STK39) (Piechotta *et al.*, 2002; Dowd & Forbush, 2003; Delpire & Gagnon, 2008). Later, from work mainly on epithelia, notably the kidney, it was found that the "with no lysine (K)" kinases (WNKs) both stimulated NKCC and inhibited KCC in a coordinate way, often working via downstream activation of SPAK/OSR1 (De Los Heros *et al.*, 2006; Kahle *et al.*, 2006; Alessi *et al.*, 2014). In RBCs, the situation remained unclear until more recently two papers have also revealed a role for WNKs in control of both KCC and NKCC. Working principally with the HEK293 cell line, but also with human red cells (Rinehart *et al.*, 2009), showed that WNK1 inhibition played a role in stimulation of red cell KCC by swelling. Latterly, Low's group has used transgenic mice to identify an excitatory role for WNK1 for OSR1

and, in regulation of the coordinate transporter, NKCC, upon deoxygenation (Zheng *et al.*, 2019).

Nevertheless, although in other tissues WNKs have been shown to modulate KCC activity, their role in mediating many of the stimuli affecting RBC KCC activity and their function in sickle cells remain poorly studied. In this chapter, the pan WNK inhibitor, WNK463, was used to assess the role of WNKs in regulation of KCC in RBCs, mainly from SCA patients but also from normal individuals, assessing its interaction with less specific pharmacological modulators of protein phosphorylation (staurosporine, NEM and calyculin A) and with the more physiologically important stimuli (pH, volume, urea, and oxygen). Results represent the first demonstration for a pre-eminent role for WNKs in modulation of KCC activity in sickle cells, suggesting a potential key target for chemotherapeutic modulation. By contrast, pharmacological results suggest that participation of the downstream kinases SPAK/OSR1 in regulation of KCC activity was lacking.

Many of these findings have been published (Lu *et al.*, 2019).

7.1 Results

7.2 Effect of WNK463 on KCl cotransport in HbSS RBCs

In the first series of experiment, the effect of the pan WNK inhibitor WNK463 was examined in RBCs from both normal individuals (termed HbAA cells) and patients with SCA (termed HbSS cells). Initially, its concentration dependence was investigated. WNK463 increased KCC activity in both HbAA and HbSS cells, with an EC_{50} of 7.9 ± 1.2 nM and 10.9 ± 1.6 nM, respectively (Figure 7.1a). The time course of the effect of WNK463 was also determined. After equilibrating in the presence of WNK463 in fully oxygenated conditions for 20 min, the inhibitor significantly increased KCC activity by about fivefold (Figure 7.1b). With additional periods of pre-incubation prior to oxygen tension equilibration, the stimulatory effect of WNK463 increased, becoming maximal after 20 min pre-incubation. On the basis of these preliminary experiments, a concentration of 40 nM WNK463 and a pre-incubation time of 30 min were chosen for subsequent work. In the absence of Cl^-, in N-MBS, K^+ influxes were 0.65 ± 0.10 and 0.70 ± 0.20 mmol.(l cells.h)$^{-1}$ in normal and sickle red cells in the absence of WNK463 and 0.74 ± 0.10 and 0.78 ± 0.15 in its presence (40 nM), means ± S.E.M., n = 3 (all n.s.), confirming that the major effect of WNK463 was mediated via KCC activity. The following is largely restricted to work in red cells from SCA patients, but similar findings were obtained with those from normal HbAA individuals and are given in brief.

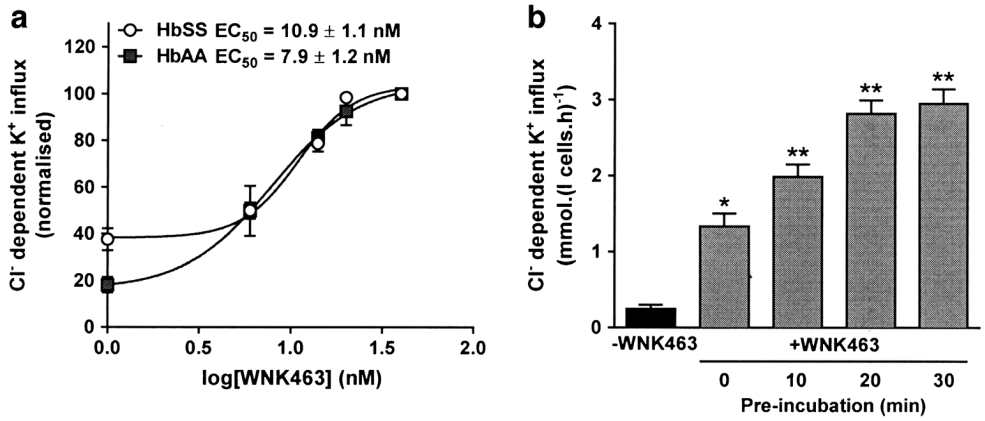

Figure 7.1 Effect of WNK463 on KCl cotransport (KCC) activity in RBCs from normal individuals (HbAA) and patients with sickle cell anaemia (SCA). RBCs from patients homozygous for SCA (20% haematocrit, Hct) or healthy individuals (40% Hct) were pre-incubated in N-MBS for 30 min at 37 °C in air in the presence of 0–40 nM WNK463, unless stated otherwise. They were then equilibrated in Eschweiler tonometers for 20 min in air (150 mmHg O_2) in the continued presence of WNK463, after which aliquots were diluted tenfold into flux tubes. KCC activity was measured as Cl^--dependent K^+-influx for 10 min at an extracellular [K^+] of 7.5 mM. KCC activity is given in mmol.(l cells.h)$^{-1}$. Ouabain (100 µM) and bumetanide (10 µM) were present in all experiments. (a) Effect of 0–40 nM WNK463 on KCC activity. KCC activity was normalised to that at 40 nM WNK463 and EC_{50} calculated using nonlinear regression. (b) Effect of duration of pre-incubation with 40 nM WNK463 on KCC activity in HbSS RBCs. Symbols represent means ± S.E.M., n = 3. * $p < 0.05$, ** $p < 0.01$ compared to RBCs incubated in the absence of WNK463.

7.3 Effect of combinations of WNK463 and staurosporine, *N*-ethylmaleimide (NEM), and calyculin A in HbSS RBCs and HbAA RBCs

Staurosporine (100 µM) represents one of the main protein kinase (PK) inhibitors used to stimulate KCC activity in RBCs (Cossins *et al.*, 1994). Its effects were compared with those of WNK463 (Figure 7.2). When incubated with each PK inhibitor alone, the stimulatory effects of WNK463 and staurosporine were similar, albeit slightly greater for WNK463. Sequential application of the two inhibitors also gave similar levels of activity although addition of WNK463 before staurosporine appeared to slightly increase KCC activity further compared to either inhibitor alone. However, the actual increase of KCC activity was only 10.6 ± 5.1% compared to WNK463 alone, suggesting a similar target kinase for both reagents.

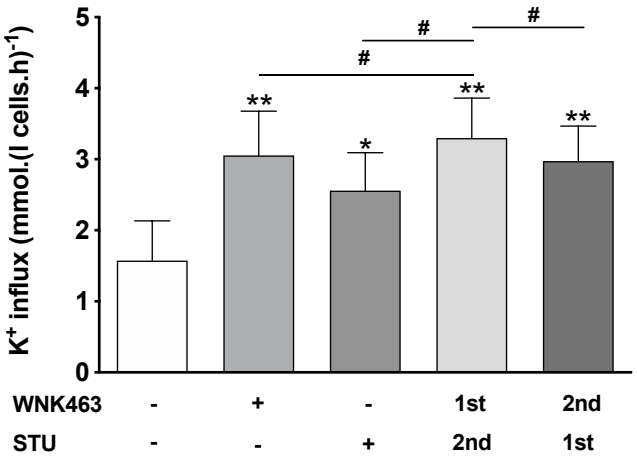

Figure 7.2 Effect of staurosporine and WNK463 on KCC activity in RBCs from patients with SCA. RBCs (20% Hct) were pre-incubated in N-MBS sequentially for two periods of 30 min in the presence of vehicle (DMSO) or drug (WNK463 40 nM or staurosporine 100 µM), as indicated. They were then equilibrated in Eschweiler tonometers for 20 min at 150 mmHg in the continued presence of WNK463 and/or staurosporine and KCC activity measured as described in the legend to Figure 7.1. Histograms represent means ± SEM, n = 4. * $p < 0.05$, ** $p < 0.01$ compared to RBCs incubated in the absence of WNK463; # $p < 0.05$ between groups as indicated.

A second putative PK inhibitor used to stimulate KCC activity in RBCs has been the thiol-reacting reagent *N*-ethylmaleimide (NEM; 100 µM) (Lauf & Theg, 1980; Hume *et al.*, 2000). When compared with WNK463, NEM alone or in combination with WNK463, NEM gave significantly lower levels of KCC activity compared to WNK463 alone, whether applied prior to or after WNK463 (Figure 7.3). Again, there was no indication of additive effects of the two reagents. As NEM/WNK463 combinations always reduced KCC activity below that of WNK463 alone, it suggested that effects other than PK inhibition were present.

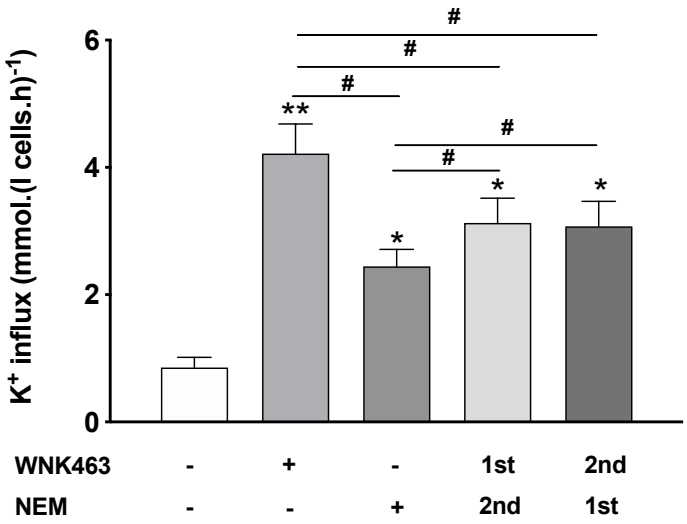

Figure 7.3 Effect of *N*-ethylmaleimide (NEM) and WNK463 on KCC activity in RBCs from patients with SCA. RBCs (20% Hct) were pre-incubated in N-MBS sequentially for two periods of 30 min in the presence of vehicle (DMSO) or drug (WNK463 40 nM or NEM 1 mM), as indicated. They were then equilibrated in Eschweiler tonometers for 20 min at 150 mmHg in the continued presence of WNK463 and/or NEM and KCC activity measured as described in the legend to Figure 7.1. Histograms represent means ± S.E.M., n = 4. * $p < 0.05$, ** $p < 0.01$ compared to RBCs incubated in the absence of WNK463; # $p < 0.05$ between groups as indicated.

The protein phosphatase inhibitor calyculin A (100 nM) has been shown to inhibit KCC activity in RBCs and to prevent subsequent stimulation by PK inhibitors following pre-incubation with calyculin A (Starke & Jennings, 1993). These observations suggest that dephosphorylation of the regulatory site controlling KCC activity involves a calyculin A-sensitive phosphatase PP1 and PP2a (Bize *et al.*, 1999, 2000). As observed previously, calyculin A on its own inhibited KCC activity below that in control cells (Figure 7.4). Following WNK463 addition, however, calyculin A had minimal effect. When added prior to WNK463, calyculin A greatly reduced the stimulatory effect of subsequent addition of WNK463. These findings are consistent with the WNK phosphoresidue target also being dephosphorylated by a calyculin A-sensitive phosphatase.

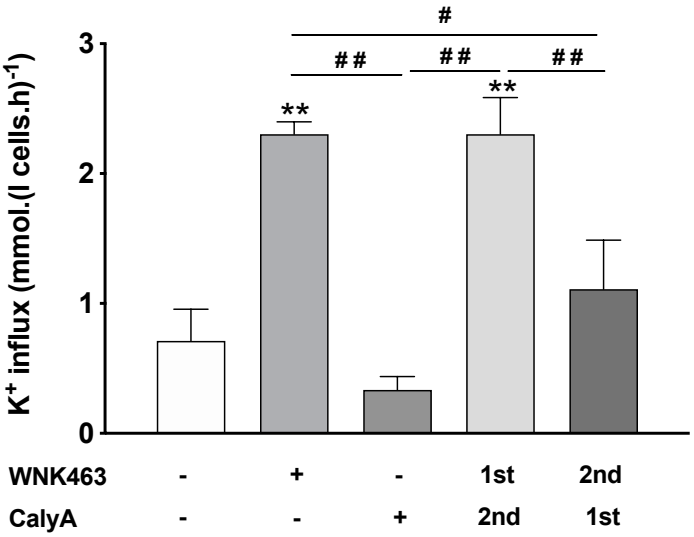

Figure 7.4 Effect of calyculin A and WNK463 on KCC activity in RBCs from patients with SCA. RBCs (20% Hct) were pre-incubated in N-MBS sequentially for two periods of 30 min in the presence of vehicle (DMSO) or drug (WNK463 40 nM or calyculin A 100 nM), as indicated. They were then equilibrated in Eschweiler tonometers for 20 min at 150 mmHg in the continued presence of WNK463 and/or calyculin A and KCC activity measured as described in the legend to Figure 7.1. Histograms represent means ± SEM, n = 6. ** $p < 0.01$ compared to RBCs incubated in the absence of WNK463; # $p < 0.05$, ## $p < 0.01$ between groups as indicated.

Similar findings were obtained with RBCs from normal individuals (HbAA). Control K^+ influxes in the absence of protein kinase/phosphatase inhibitors were 0.2 to 0.3 mmol.(l cells.h)$^{-1}$. In the presence of staurosporine (100 μM) and NEM (1 mM), these increased to 3.41 ± 0.86 and 3.01 ± 0.60 mmol.(l cells.h)$^{-1}$, respectively. When treated with combination of staurosporine or NEM and WNK463 (40 nM), influxes were 3.5 ± 0.5 and 3.55 ± 0.80 mmol.(l cells.h)$^{-1}$ (means ± S.E.M, n = 3; N.S. cf staurosporine and NEM alone), respectively – showing that the action of WNK463 and staurosporine/NEM was not additive. With calyculin A (100 nM), influxes were reduced from 0.30 ± 0.07 to 0.20 ± 0.06 mmol.(l cells.h)$^{-1}$ increasing to 3.40 ± 0.82 with WNK463 alone, and in combination with WNK463 after calyculin A, they were 0.43 ± 0.16 mmol.(l cells.h)$^{-1}$, showing that pre- treatment of RBCs with the protein phosphatase inhibitor calyculin A prevented KCC in normal RBCs from responding to WNK463.

7.4 The effect of combinations of WNK463 and physiological stimuli modulating KCC activity in HbSS cells

The effect of changes in pH and volume change and also incubation with high concentrations of urea was compared in control HbSS cells and following pre-incubation with WNK463. All three stimuli significantly elevated KCC activity – as shown previously (Gibson & Ellory, 2003) – but none stimulated activity to the extent achieved by WNK463 alone (Figure 7.5a–c). Notwithstanding all three were still able to increase KCC activity following pre-incubation with WNK463, although the fold changes in activity were considerably reduced compared with those in cells not pre-incubated with WNK463 (Figure 7.5d). These findings may indicate that whilst these other stimuli may act mainly through WNK inhibition, their effect must also be mediated via some other mechanism, as suggested for the coordinate transport NKCC in HEK293 cells (Hannemann & Flatman, 2011). Conversely, it may be that pre-incubation was insufficient to completely abrogate WNK activity. A similar pattern was also found in normal HbAA RBCs.

In the case of RBC NKCC, WNK1 appears to be responsible for increased activity following deoxygenation (Zheng *et al.*, 2019). KCC activity is also oxygen dependent, although in a reciprocal fashion being activated by oxygenation rather than deoxygenation. In RBCs from normal individuals, KCC is maximally active under conditions of full oxygenation, with activity declining as oxygen tension is lowered such that the transporter is inactive when cells are fully deoxygenated (Gibson *et al.*, 1998). In sickle cells, KCC activity has an abnormal oxygen dependence, with highest activity in fully oxygenated and fully de-oxygenated cells with a nadir at about the PO_2 required for half maximal saturation of Hb with oxygen (Gibson *et al.*, 1998). This abnormal oxygen dependence was confirmed here (Figure 7.6). The interaction of WNK and oxygen tension was also investigated. When pre-incubated with WNK463, KCC activity was maximally stimulated and became insensitive to changes in oxygen tension. These findings suggest a role for WNK in mediating the oxygen sensitivity of KCC, as well as for that of NKCC.

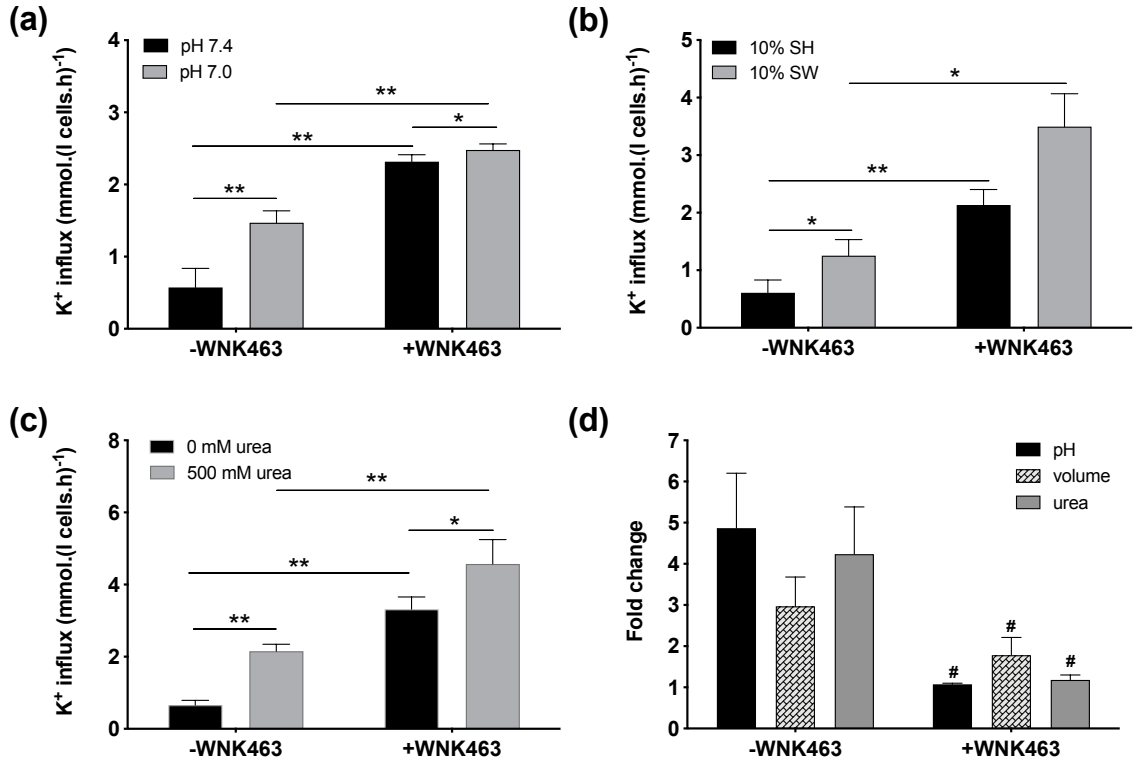

Figure 7.5 Effect of WNK463 on physiological stimuli of KCC in RBCs from patients with SCA. RBCs (20% Hct) were pre-incubated in N- MBS in the presence or absence of WNK463 (40 nM) at 37 °C in air. They were then equilibrated in Eschweiler tonometers for 20 min at 150 mmHg, after which aliquots were diluted tenfold into flux tubes containing buffers varying in pH, osmolarity, or urea content, all in the continued presence or absence of WNK463, and KCC activity was measured as described in the legend to Figure 7.1. (a) Effect of WNK463 on pH-dependent KCC activity, n = 6. (b) Effect of WNK463 on KCC activity in 10% shrunken (SH) or 10% swollen (SW) RBCs, n = 5. (c) Effect of WNK463 on urea-induced KCC activity, n = 6. (d) Impact of WNK463 on pH, volume, and urea-stimulated KCC activity. Histograms represent means ± SEM of n individual samples. * $p < 0.05$, ** $p < 0.01$ compared to indicated condition, # $p < 0.05$ compared to fold change in absence of WNK463.

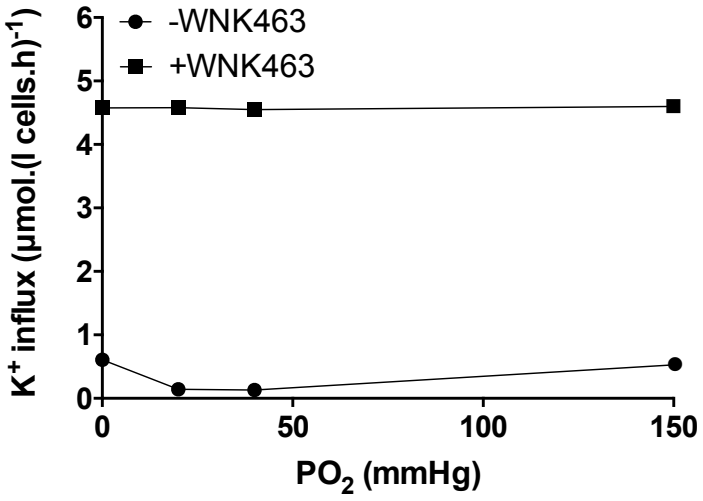

Figure 7.6 Effect WNK463 on oxygen-dependent KCC activity in RBCs from patients with SCA. RBCs (20% Hct) were pre-incubated in N-MBS in the presence or absence of WNK463 (40 nM) at 37 °C in air. They were then equilibrated in Eschweiler tonometers for 20 min at 0–150 mmHg, after which aliquots were diluted tenfold into flux tubes, all in the continued presence or absence of WNK463, and KCC activity was measured as described in the legend to Figure 7.1. Symbols represent means ± S.E.M. (error bars are smaller than symbols), n = 6.

7.5 The effect of inhibitors of SPAK/OSR1

In many cases, SPAK/OSR1 are implicated as a downstream target of WNKs. Following phosphorylation by WNKs, SPAK/OSR1 then carry out phosphorylation of the relevant CCC. The effects of several SPAK/OSR1 inhibitors (STOCK2S-26016, closantel, and rafoxanide) as well as of HK01, an inhibitor of MO25, a scaffolding protein that increases SPAK/OSR1 activity > 100-fold, were investigated. None of these inhibitors, however, gave comparable effects to that of WNK463. STOCK2S-26016 and HK01 did stimulate a Cl^--dependent K^+ influx, but effects were minimal (Figure 7.7a and b for healthy HbAA RBCs) whilst 40 nM WNK463 increased it about tenfold, from 0.35 ± 0.04 to 3.1 ± 0.2 mmol.(l cells.h)$^{-1}$ (n = 24). In comparison, in RBCs from HbSS patients Cl^--dependent K^+-influx increased from 0.48 ± 0.2 to 1.31 ± 0.3 mmol.(l cells.h)$^{-1}$ in the presence of HK01 ($p < 0.021$; n = 3) and from 0.70 ± 0.10 to 3.3 ± 0.2 mmol.(l cells.h)$^{-1}$ (n = 3) in the presence of 40 nM WNK463. Both closantel and rafoxanide also increased K^+ influx, but in this case, transport was not Cl^--dependent suggesting a nonspecific increase in membrane permeability rather than stimulation of KCC (data not shown). These findings are evidence against a major role for SPAK/OSR1 in the phosphorylation pathway modulating red cell KCC activity.

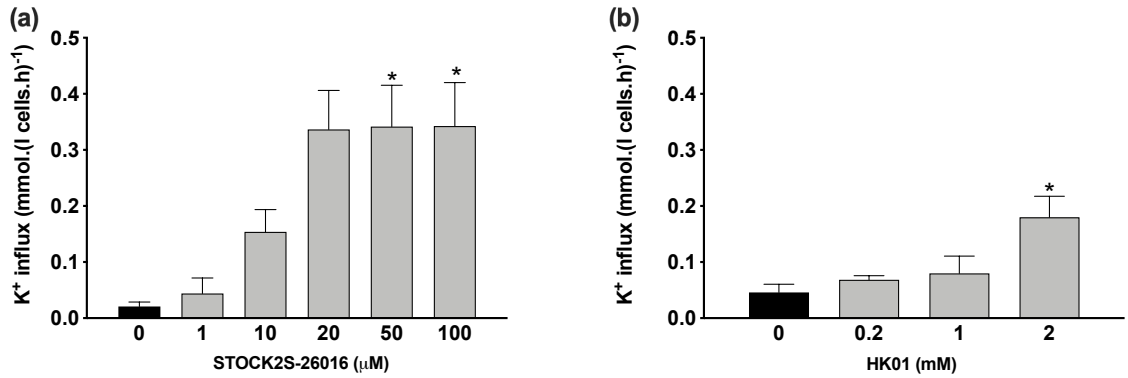

Figure 7.7 Effect of SPAK/OSR1 inhibitors on KCC activity in HbAA RBCs. RBCs (40% Hct) were pre-incubated in N-MBS in the presence of 0– 100 μM STOCK2S-26016 or 0–2 mM HK01 at 37 °C in air. They were then equilibrated in Eschweiler tonometers for 20 min at 0–150 mmHg, after which KCC activity was measured as described in the legend to Figure 7.1. (a) Effect of STOCK2S-26016 on KCC activity in HbAA cells, n = 3. (b) Effect of HK01 on KCC activity in HbAA cells, n = 3. Histograms represent means ± SEM of n individual samples. * $p < 0.05$.

7.6 Discussion and conclusion

RBC KCC is sensitive to a number of physiological stimuli including pH, urea, oxygen tension and volume (Gibson & Ellory, 2003). These modalities appear to affect the transporter by protein (de)phosphorylation (Jennings & Al-Rohil, 1990; Jennings & Schulz, 1991), with pharmacological evidence for the presence of both serine/threonine and tyrosine phosphoresidues (Cossins *et al.*, 1994; Bize *et al.*, 1999). This chapter provides additional demonstration of a functional role for WNKs in control of KCl cotransport in RBCs from SCA patients and normal individuals.

The present work makes use of the pan WNK inhibitor, WNK463. This inhibitor produced a marked activation of KCC with an EC_{50} of around 10 nM, which is about the concentration reported in the literature for a specific effect on WNKs. The EC_{50} of WNK463 for different WNKs varies and is reported to be 5, 1, 6, and 9 nM for WNKs 1, 2, 3 and 4, respectively (Yamada *et al.*, 2016). The functional assays of KCC activity, however, are insufficiently sensitive to use these small concentration differences to identify the main RBC WNK. An important caveat, however, is that definitive proof of the role of WNKs awaits phosphorylation studies in mature red cells or knockdown assays in nucleated RBC precursors. Nevertheless, as the abnormally high activity of KCC in RBCs from sickle cell patients is known to mediate solute loss and decrease in cell volume, KCC stimulation induced by WNK463 would be expected to result in shrinkage.

Both hypertonicity and a reduction of internal [Cl^-] have been shown to activate WNKs (Xu *et al.*, 2000; Zagórska *et al.*, 2007; Richardson & Alessi, 2008) leading to an inhibition of KCC activity. In addition, hypotonicity, sometimes in combination with high [K^+], had the opposite effect, decreasing WNK phosphorylation and activating KCC (De Los Heros *et al.*, 2006; Zhang *et al.*, 2016). Here, hypotonicity, low pH, and urea were used to increase KCC activity which was increased further by treatment with WNK463. Notwithstanding, the sensitivity to these physiological stimuli was significantly reduced in the presence WNK463, consistent with the involvement of WNKs, and widening the stimuli with which these enzymes are associated.

Oxygen is another important physiological regulator of KCC in RBCs. Until recently the mechanism was unknown although Hb represented the most obvious sensor (Gibson *et al.*, 2000). DeoxyHb has profound effects on red cell function, acting largely through

its greater affinity for the cytoplasmic tail of band 3 (AE1) compared to oxyhaemoglobin and from which it displaces several proteins, including several glycolytic enzymes and ankyrin (Low *et al.*, 1993; Chu *et al.*, 2016). In a similar way, deoxyHb was also recently found to compete with WNK1 following which its release into the cytoplasm led to OSR1 activation and subsequent NKCC1 phosphorylation and activation (Zheng *et al.*, 2019). As NKCC and KCC are often regulated reciprocally, activation of WNK1 by deoxygenation would explain phosphorylation and inactivation of KCC. In SCA, however, KCC activity is abnormally high, and its aberrant response to deoxygenation – an increase in activity as oxygen tension falls from the P_{50} of Hb to 0 mmHg – may be explained if polymerisation of HbS removes the source of deoxyHb for WNK displacement leading to decreased WNK activity. The present findings show that inhibiting WNK with WNK643 not only significantly increased KCC activity but also abrogated its oxygen dependence.

Other pharmacological inhibitors of protein phosphorylation also increased KCC activity. Staurosporine, which interacts with over two hundred and fifty human kinases with varying potency, showed a similar effect to WNK463, whilst a combination of both did not appear to be additive. Moreover, it has been shown to inhibit WNK1 directly in vitro in an ATP-dependent manner (Yagi *et al.*, 2009), providing a possible mechanism for its observed action on KCC activity and lack of any additive effects. NEM acts via modification of sulfhydryl group of cysteine residues and has been shown to decrease phosphorylation at an established WNK phosphorylation site in SPAK required for its activity, as well as at the T1048 equivalent in KCC2 leading to increased activity in KCC2 transfected HEK293 cells (Conway *et al.*, 2017). Whilst KCC2 is not present in red cells, these findings suggest a possible mechanism for KCC activation by NEM. Finally, the effect of WNK inhibition was largely abrogated by pre-treatment with calyculin A, indicating a role for protein phosphatases PP1 and PP2A in dephosphorylation of the WNK target.

In conclusion, the present findings confirm the involvement of WNK in negative regulation of KCC activity in human RBCs. Whilst this is not a surprise and has been shown for several tissues, notably epithelia and neurons, there is little information on its role in RBCs aside from a single report on hypotonically induced KCC activity and, to date, nothing on sickle cells. In addition, results further emphasise the role of WNKs

in influencing KCC activity by important physiological modulators – volume, pH, urea, and oxygen tension – extending our understanding of the mechanisms by which KCC in RBCs is controlled.

8 General discussion

Since the discovery of SCD by Dr James Herrick in 1910, a plethora of studies on the pathogenesis and its protean complications have been suggested. These low-cost SCD treatment strategy plans have largely contributed to a longer life span for many SCD patients, especially in some developing countries. Hydroxyurea as one of the more reliable treatments on SCD, has been approved for use on adults, while the use of HU in young SCA patients is usually restricted to more severely affected children (Rees, 2011). Moreover, more than 30% SCA patients show no response to HU, while in others its use it limited because of several deleterious side effects (Strouse & Heeney, 2012). Therefore, it is important to find out other affordable and milder side effect of therapeutic strategies for the treatment of SCD.

HbS polymerisation and sickling, increased PS exposure and aberrant activity of KCl cotransport are critical to the pathogenesis of SCD. However, there is more than one factor that affects their behaviour. This thesis mainly addresses a number of physiological stimuli and pharmacological stimuli / reagents which contribute to sickling, PS exposure and KCC activity, together with potential strategies of inhibiting these effects. A better understanding of the role of physiological / pharmacological factors controlling these events would inform the development of better SCD therapeutic strategies in the future.

8.1 Role of physiological factors in the pathogenesis of SCD

Taken together, the relative significance of various physiological factors on HbS polymerisation, PS exposure and KCC activity were addressed. Table 8.1 summarises the effect of these physiological factors. Various *in vitro* physiological stimuli which, such as PO2 per se, rate of deoxygenation, hydrogen ion concentration (pH), urea, anisotonicity, medium composition and duration of conditions, have been investigated in Chapters 4 - 6.

The root cause of SCD arises from the polymerisation of HbS to form fibres and distort the RBC membrane, eventually resulting in the inability of RBCs to deform effectively in the microcirculation, eventually resulting in irreversibly sickled cells, and compromising their rheological ability. The lesion of RBC membrane caused by the polymerisation of HbS causes them to become rigid, fragile and distorted, which

further activates the deoxygenated-induced cation conductance (P_{sickle}). The activation of P_{sickle} allows entry of Ca^{2+} and loss of Mg^{2+}, which induces the externalisation of PS, activates the Gardos channel and affects KCC activity. Furthermore, loss of K^+ / Cl^- and water, via Gardos channel or KCC activation, leads to cell dehydration, shrinkage and death. There is a role for band 3 in these events with altered binding of HbS to its N-terminal of band 3 causing further permeability abnormalities (Gibson & Ellory, 2002). Also, rate of deoxygenation can alter the extent of sickling, PS exposure and KCC activity in HbSS RBCs.

Regulation of extracellular pH is also important to SCD pathogenesis and management. Low pH commonly occurs in metabolic acidosis SCA patients. HbS molecules have a decreased solubility at lower pH, with a resulting increase in the driving force for HbS polymerisation and P_{sickle} activity (Ueda & Bookchin, 1984) and elevation of KCC activity. Ca^{2+} entry and Mg^{2+} loss will further increase externalisation of PS and activation of KCC, contributing to pathogenesis.

Urea supplementation was proposed by Murayama's group in 1971 as a potential therapy for SCD. The mechanism of sickling involves the formation of intermolecular hydrophobic bonds resulting in the polymerisation of HbS and the formation of fibres (Murayama, 1966). Urea *in vitro* plays in a role in inhibiting the gelation of deoxygenated sickle HbS via a chemical attack on these hydrophobic bonds, preventing sickling and providing a successful and less toxicity treatment for SCD (Javid, 1958). Therefore, the prevention of forming HbS fibres leads to inhibit PS exposure and its sequalae. However, findings in Chapter 6 suggests urea plays a role in stimulating KCC-mediated volume reduction in HbSS RBCs (Joiner *et al.*, 2007). In section 6.3, urea is shown to stimulate oxygen-dependent KCC activity at PO_2=100 mmHg. According to Joiner *et al.*, urea stimulates volume-dependent KCC activity in HbSS RBCs as well. These results suggest urea may not be a perfect treatment to cure SCD, accompanying with the risk of the activation of KCC in HbSS RBCs. On the other hand, urea reduces sickling and PS exposure. Its potential benefit therefore requires further re-evaluation.

Cell volume is another physiological stimulus which may determine the degree of sickling, PS exposure and KCC activity in HbSS RBCs. The effect of intracellular [HbS] may be manipulated by the osmotic conditions surrounding the RBCs. Hypotonic saline

solutions decrease sickling and KCC activity in HbSS RBCs - Chapter 4 and Chapter 6. Previous studies have shown the benefit of reducing plasma osmolality in patients (Rosa *et al.*, 1980; Carden *et al.*, 2017) and this is still carried out today (Rees, personal communication). The present work provides a greater rational basis for its use in clinical patients. However, the risk of activation of KCC in HbSS RBCs represents an important caveat.

Table 8.1 Effect of physiological stimuli on sickling, PS exposure and KCC activity in HbSS RBCs.

Physiological stimuli	**Sickling**	**Phosphatidylserine (PS) exposure**	**KCC activity**
PO_2	PO_2 ↓, sickling ↑	PO_2 ↓, PS ↑, in the presence of extracellular Ca^{2+} (Weiss *et al.*, 2011)	PO_2 ↓, KCC ↑ (Joiner & Franco, 2001)
Rate of deoxygenation	Rate ↑, sickling ↑	Rate ↑, PS ↑	NT*
pH	pH ↓, sickling ↑	pH ↓, PS ↑	pH ↓, KCC ↑
Urea	Sickling ↓	PS ↓	KCC ↑
Anisotonicity	Cell volume ↑, sickling ↓	NT*	Cell volume ↑, KCC ↑
Medium composition	Compared to Cl-MBS, sickling ↓ in BBS and plasma	Compared to Cl-MBS, PS ↓ in BBS and plasma	Compared to Cl-MBS, KCC ↓ in BBS and plasma
Duration of conditions	When PO_2 ↓ and time ↑, then sickling ↑ (pH 8.0 - pH 6.5)	NT*	NT*
Temperature	Temperature ↑, sickling ↑ (Iqbal *et al.*, 2013)	Temperature ↑, PS ↑ (Zhang *et al.*, 2018)	Temperature ↑, KCC ↑ (Khan & Ellory, 2000)
Cell age	Age ↑, sickling ↑ (Papageorgiou *et al.*, 2018)	No effect after short-time incubation (Wesseling *et al.*, 2016)	Age ↑, KCC ↑ (Hannemann *et al.*, 2015)

*Not tested

8.2 Role of pharmacological factors in the pathogenesis of SCD

Table 8.2 summarises the effect of these pharmacological factors on the three major facets of the pathogenesis of SCD: HbS polymerisation and sickling, PS exposure and KCC activity.

Staurosporine acts a protein kinase inhibitor which is utilised to stimulate KCC activity in RBCs. As shown in Table 8.2, staurosporine had no effect on sickling but stimulated KCC activity, so similar compounds may therefore not be a good pharmacological approach to treat SCD. Calyculin A is a protein phosphatase inhibitor that inhibits KCC but had no effect on sickling. WNK463 is pan WNK inhibitor, which can stimulate KCC activity but again had no effect on sickling. In Chapter 4, cytochalasin B acting as an inhibitor of the binding of G-actin to the RBC membrane and cytochalasin B also inhibits sugar transporter which shows no effect on sickling. EDTA is an anticoagulant reagent in blood sample, which had an inhibitory effect on PS exposure, while its effects on sickling and KCC activity remain to be investigated. A spider venom neuropeptide, GsMTx-4, and ruthenium red increased PS exposure in HbSS RBCs, although it is likely that these actions were due to some non-specific effect. Their effects on sickling and KCC activity were not studied. STOCK2S-26016 and HK01 are two SPAK/OSR1 inhibitors which had a modest stimulatory effect on stimulate KCC activity, while their effects on sickling and PS exposure remain to be investigated. In previous work in HEK293 cell lines, a major role for WNK1 was implicated in control of KCC3 activity using RNA interference (RNAi), with less evidence for WNK2 and WNK4 (Rinehart *et al.*, 2009). In these cells, WNK1 inhibition activated volume-sensitive KCC3 activity via dephosphorylation of T991 and T1048. The same residues were dephosphorylated in hypotonically induced KCC3 activity in red cells, although no evidence was presented for which WNK was involved. Phosphorylation of KCC3 T1048 – and its equivalent in the other KCC isoforms – was later shown to be mediated by SPAK/OSR1 whilst that of T991 was not (De Los Heros *et al.*, 2006; Zhang *et al.*, 2016), with neither residue being directly phosphorylated by WNK1 or WNK3. In the present study, several SPAK/OSR1 inhibitors with different mechanisms of action were tested (STOCK2S-26016, HK01, closantel, and rafoxanide), but their impact on RBC KCC activity was minimal.

Moreover, this apparent paradox could be explained by several observations. When either T991 or T1048 were mutated to alanine, KCC activity increased moderately and could still be modulated by low internal [Cl^-] or volume change. By contrast, T991A/T1048A double mutants were highly active, and the incubation medium had no further impact. Furthermore, *in vitro* phosphorylation experiments using purified KCC and SPAK or OSR1 showed phosphorylation of T1048 but not T991 (De Los Heros *et al.*, 2006; Rinehart *et al.*, 2009), whilst in an ES knock-in model lacking SPAK and OSR1 activity, only T991 was phosphorylated (De Los Heros *et al.*, 2006). The effect of a siRNA knockdown of WNK1 in HEK293 was less clear. It markedly reduced KCC phosphorylation, whilst knockdown of SPAK or OSR1 did not (Rinehart *et al.*, 2009). It is unclear, however, if the knockdowns of SPAK and OSR1 were simultaneous or separate. If the latter was the case, then one enzyme could have compensated for the loss of the other. Should SPAK/OSR1 only be involved in the phosphorylation of T1048 but that of both T991 and T1048 are required for a full impact on KCC regulation, inhibiting SPAK/OSR1 would be expected to have a much smaller effect than inhibiting upstream WNK. Further understanding of the pathways controlling KCC in red cells awaits identification of the phosphoresidues involved – and is proposed as an important area for future work.

Overall, Figure 8.1 shows a cartoon that the effects of various physiological stimuli and pharmacological factors on sickling, PS exposure and KCC activity in HbSS RBCs.

Table 8.2 Effect of pharmacological factors on sickling, PS exposure and KCC activity in HbSS RBCs. n.s. represents "not significant".

Pharmacological factors	Sickling	Phosphatidylserine exposure	KCC activity
Staurosporine	n.s.		↑
Calyculin A	n.s.		↓
NEM	n.s.		↑
WNK463	n.s.		↑
Cytochalasin B	n.s.		
EDTA		↓	
GsMTx-4		↑	
Ruthenium red		↑	
STOCK2S-26016			↑
HK01			↑

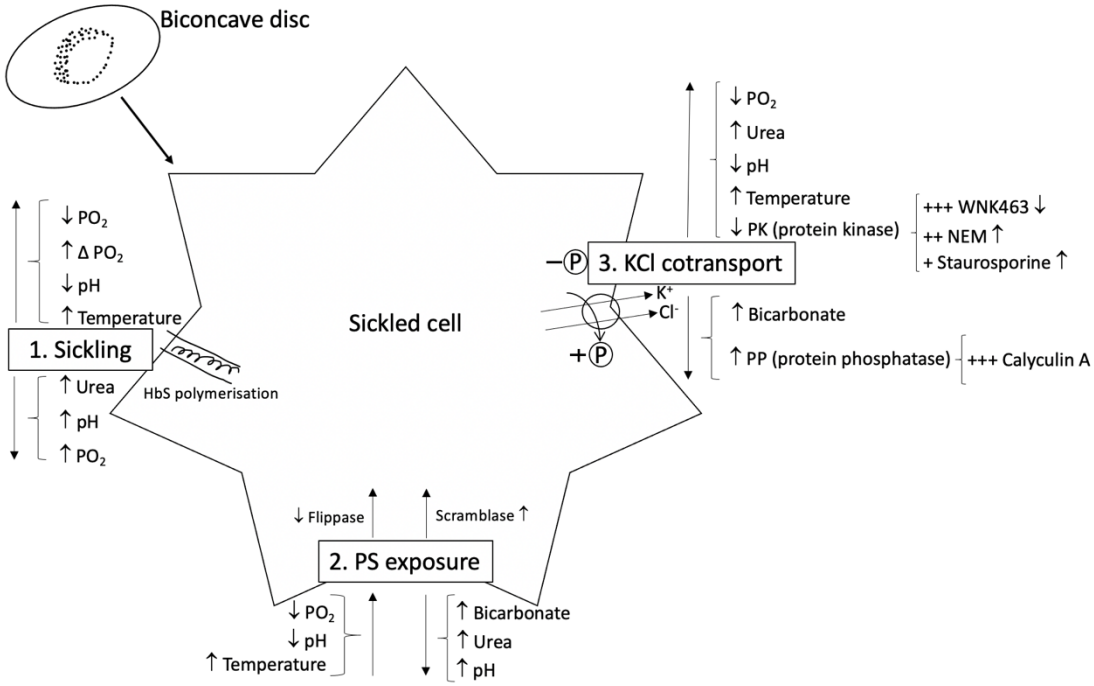

Figure 8.1 Schematic diagram to illustrate the main physiological and pharmacological factors affecting sickling, phosphatidylserine (PS) exposure and KCl cotransport (KCC) activity in red cells from patients with sickle cell disease (SCD). Sickling, PS exposure and KCC activity are all implicated in pathogenesis of SCD. Oxygen tension (PO_2), rate of deoxygenation (ΔPO_2), pH, temperature, urea and bicarbonate - each of which occurs normally in the plasma *in vivo* - all affect the extent of sickling, PS exposure and KCC activity - as illustrated - and thereby have an important impact on disease progression. In addition, inhibitors of protein phosphorylation (notably the pan-WNK kinase inhibitor, WNK463, but also N-ethylmaleimide (NEM) and staurosporine) and of protein phosphatases (calyculin A) have profound effects on KCC activity but do not alter sickling. Less is known about their effects on PS exposure, though NEM does increase lipid scrambling (Hannemann *et al.*, 2018). Rather, PS exposure is more directly controlled by protein kinase C activity (Wesseling *et al.*, 2016).

8.3 Other treatments of SCD

Newer therapeutic approaches to inhibit intravascular sickling can be classified into those inhibiting the gelation of HbS upon deoxygenation, those modifying the membrane making it less prone to sickling, inhibitors of membrane transport proteins, and those improving peripheral perfusion.

These various approaches are summarised in Table 8.3. Notwithstanding, several are not compatible with a possible clinical applicability. Hence, several covalent (such as sodium cyanate) and non-covalent inhibitors (such as Fagara zanthoxyloides) may be limited by their toxicity. Some other agents which fail to cross the RBC membrane, and cannot therefore inhibit sickling, may still inform similar reagents which may have a broader clinical application.

A successful antisickling agent should be easy to administer, and preferably amenable to administration orally. Also, it needs to cross RBC membrane and interact with HbS or interact with the RBC membrane, without serious side effects. Moreover, it is important to prevent deleterious haematological side-effects such as increased haemolysis or increased O_2 affinity. To date, few reagents in Table 8.3 have come close to satisfying these requirements. It is hoped that some of the results of this thesis may be used to inform design of better compounds. The beneficial action of urea, in particular, may be utilised to create similar membrane permeable low molecular weight compounds with similar actions at higher affinity. Likewise, the marked stimulatory effect of WNK kinase inhibitors on KCC indicates that development of reagents which are able to stimulate RBC WNK kinases may be viable.

Despite its emergence the "first molecular disease", it is clear that further research into the mechanisms and treatment of SCD remains urgently required.

Table 8.3 Other therapeutic strategies for SCD (listed by year)

Treatment	Mechanism	References
Blood transfusion	Dilution of [HbS]	(Cooley, 1927)
Acetazolamide	Increase oxygen affinity	(Hilkovitz, 1957)
Sodium bicarbonate	Increase oxygen affinity	(Greenberg & Kass, 1958)
Phenothiazines	Inhibit sickling	(Hathorn & Lewis, 1966)
Testosterone	Inhibit sickling	(Lundh & Gardner, 1970)
Sodium cyanate	Inhibit HbS polymerisation	(Gillette *et al.*, 1971)
Acetylsalicylic acid	Increase oxygen affinity	(Klotz & Tam, 1973)
Mechlorethamine	Inhibit gelation	(Roth *et al.*, 1973)
Zinc	Inhibit sickling	(Brewer & Oelshlegel, 1974)
Fagara zanthoxyloides	Inhibit gelation and sickling	(Ekong *et al.*, 1975; Poillon & Bertles, 1977)
Alkylurea	Inhibit gelation and sickling	(Elbaum *et al.*, 1976)
Aromatic amino acids	Inhibit gelation by competition for protein-protein contact sites	(Noguchi & Schechter, 1977)
Pyridoxal sulphate	Inhibit HbS polymerisation	(Kark *et al.*, 1978)
Cetiedil	Inhibit sickling	(Asakura *et al.*, 1980)
Methyl acetimidate	Inhibit sickling	(Chao *et al.*, 1981)
5-Azacytidine	Decreasing [HbS]	(DeSimone *et al.*, 1982)
Piracetam	Inhibit sickling	(Gini & Sonnet, 1987)
Pentoxifylline	Inhibit sickling	(Bilto *et al.*, 1988)
Bepridil	Inhibit sickling	(Johnston *et al.*, 1989)
H74	KCC inhibitor	(Ellory *et al.*, 1990)
p-hydroxybenzoic acid	Inhibit sickling	(Akojie & Fung, 1992)
Clotrimazole and derivatives	Gardos channel inhibitors	(Brugnara *et al.*, 1993)
Nitric oxide	Increase oxygen affinity	(Head *et al.*, 1997)
Anionic Polysaccharides	Reduce adhesion of sickle RBCs	(Barabino *et al.*, 1999)
Magnesium pidolate	KCC inhibitor	(De Franceschi *et al.*, 2000)
Monoclonal antibodies to alphaVbeta3	Reduce adehesion of sickle RBCs	(Kaul *et al.*, 2000)
ICA-17043 ("*Senicapoc*")	Gardos channel inhibitor	(Stocker *et al.*, 2003)
N-Acetylcysteine	Antioxidant	(Pace *et al.*, 2003)
Vanillin pro-drug (MX-1520)	Increase oxygen affinity	(Zhang *et al.*, 2004)
5-Hydroxymethyl-2-furfural (5HMF or Aes-103)	Increase oxygen affinity	(Safo *et al.*, 2004)

L-glutamine	Antioxidant	(Niihara *et al.*, 2005)
Polynitroxyl albumin	Inhibit VOC and inflammation	(Mahaseth *et al.*, 2005)
Eptifibatide	Anti-platelet	(Lee *et al.*, 2007)
Tinzaparin (heparin)	Reduce VOC	(Qari *et al.*, 2007)
Bosentan	Reduce VOC	(Sabaa *et al.*, 2008)
INN-298 and INN-312	Increase oxygen affinity	(Nnamani *et al.*, 2008)
Propranolol	Reduce adrenaline-induced RBC adhesion	(De Castro *et al.*, 2012)
Prasugrel	Anti-platelet	(Wun *et al.*, 2013)
TD-1	Increase oxygen affinity	(Nakagawa *et al.*, 2014)
Voxelotor (GBT440)	Increase oxygen affinity	(Oksenberg *et al.*, 2016)
GBT1118	Increase oxygen affinity	(Dufu *et al.*, 2017)
Adakveo (crizanlizumab-tmca)	Inhibition of cell adhesion	(Ataga *et al.*, 2017)

9 Conclusion

One of the aims of this study was a better understanding of the physiological stimuli involved in SCD pathogenesis, acting directly on the sickle RBC, and mediated by HbS polymerisation, PS exposure and KCC activity, together with an investigation of possible pharmacological factors which may ameliorate the complications of SCD mediated by such changes.

The polymerisation of HbS in sickle cells is the underlying cause of all the complications of SCD. Chapter Four of this thesis shows various physiological stimuli such as PO_2, rate of deoxygenation, pH, urea, anisotonicity, medium composition clearly affect the extent of polymerisation of HbS and sickling. Furthermore, although some pharmacological reagents such as staurosporine, calyculin A, NEM, WNK463 and cytochalasin B, showed no effect on HbS polymerisation and sickling, they might be useful in modulation of protein phosphorylation and thus of KCC activity, which will remain an area for future investigation.

A further critical part of SCD pathogenesis is PS exposure, as along with HbS polymerisation and the sickling shape change, it will also promote microvascular occlusion. Chapter Five of this thesis shows that various physiological stimuli such as rate of deoxygenation, pH, urea, medium composition clearly affect the extent of externalisation of PS, and may represent valuable avenues for future study.

Altered membrane cation permeability is also key to pathogenesis as it causes shrinkage. The relationship between KCC and some of its physiological modulators was considered in Chapter 6. Again, pH, urea, volume and medium composition are key physiological stimuli which can regulate KCC in HbSS RBCs.

Finally, the aim of Chapter 7 was to investigate the role of WNK/SAPK/OSR1 pathway in sickle cells. A major stimulatory effect of the pan WNK inhibitor - WNK463 - indicates a predominant role for WNK kinases in control of RBC KCC activity. In Chapter 7, some other kinases inhibitors such as staurosporine, NEM, calyculin A were utilised in combination with WNK463 to explore their roles in WNK/SAPK/OSR1 pathway. Results indicated a hierarchy of control with WNK kinases predominant in regulation of KCC. A more complete understanding of the mechanisms will inform pathogenesis whilst manipulation of WNK activity represents a potential therapeutic approach for SCD.

10 Future work

1. To have a better understanding of the role of KCC phosphoresidue on the regulation of KCC, with the possible use of quantitative phosphoproteomics.

2. Use of CRISPR/cas9 and immortalised erythroid cells lines (Bristol Erythroid Line Adult; BEL-A) to develop a system amenable to molecular intervention.

3. Use of CRISPR/cas9 shRNA to knockout/knockdown *PIEZO1* and *SCL4A1* (band 3) in each SCD cell lines and in control BEL-A cells without the sickle mutation, to identify key molecular mechanisms involved in regulation of KCC activity and probe the identity of P_{sickle}. Such studies may inform design of newer, effective treatments.

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